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Molecular characterization of porcine respiratory coronavirus isolates with varying pathogenicity

by

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LIST OF ABBREVIATIONS

AcNPV	*Autographa californica* Nunclear Polyhedrosis Virus
[α ^{32}P]dCTP	alpha phosphorous-32 deoxycytosine triphosphate
APN	aminopeptidase N
bp	base pairs
CCV	canine coronavirus
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CPE	cytopathic effect
DEAE-dextran	diethylaminoethyl dextran
dCTP	deoxycytosine triphosphate
dUTP	deoxyuridine triphosphate
DPI	days post inoculation
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
FIPV	feline infectious peritonitis virus
FBS	fetal bovine serum
HA	hemagglutitin
HCl	hydrochloric acid
HDCD	hysterectomy-derived colostrum-deprived
HE	hemagglutination-esterase
IFA	indirect immunofluorescence assay
IgA	immunoglobulin A
kb	kilobase

kDa	kiloDalton
M	integral membrane
M	molar
MAb	monoclonal antibody
MEM	minimum essential medium
MHV	mouse hepatitis virus
M_r	molecular mass
mRNA	messenger ribonucleic acid
µCi	microCuries
µg	microgram
µl	microliter
ml	milliliter
mm	milliimeter
mM	millimolar
mU	milliunits
N	normal
N	nucleoprotein
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
nt	nucleotide(s)
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PEG	polyethylene glycol
pfu	plaque forming units
pg	picograms
p.i.	post infection
PRCV	porcine respiratory coronavirus
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
S	spike
SDS	sodium dodecyl sulfate
Sf9	*Spodoptera frugiperda* ovary
SP	small plaque mutant
SPF	specific-pathogen-free
sM	small intergral membrane
ST	swine testicular
SSC	sodium chloride-sodium citrate buffer
SSPE	sodium chloride-sodium phosphate-ethylenediamine tetraacetic acid buffer
TAE	tris[hydroxymethyl aminomethane]-acetate-ethylenediamine tetraacetic acid buffer
TCID	tissue culture infectious dose
TE	tris[hydroxymethyl aminomethane]-ethylenediamine tetraacetic acid buffer
TGE	transmissible gastroenteritis
TGEV	transmissible gastroenteritis virus
TLM83	transmissible gastroenteritis virus-like mutant
TNE	tris[hydroxymethyl aminomethane]-sodium chloride-ethylenediamine tetraacetic acid buffer

Tris	tris[hydroxymethyl aminomethane]
Tris-HCl	tris[hydroxymethyl aminomethane]-hydrochloric acid

GENERAL INTRODUCTION

Dissertation Organization

This dissertation contains three separate manuscripts. Each individual manuscript is complete in itself and includes an Abstract, Introduction, Materials and Methods, Results, Discussion, and Reference section. The Ph.D. candidate, Eric Martin Vaughn, is the senior author and principal investigator for each manuscript. The first manuscript describes the isolation and preliminary characterization of new porcine respiratory coronavirus (PRCV) isolates and has been submitted for publication in the *Journal of Clinical Microbiology*. The second manuscript, which has yet to be submitted for publication, describes the molecular characterization of new PRCV isolates and the potential role of coronavirus genes in pathogenesis and virulence. The third manuscript describes the use of nonradioactive cDNA probes in a nucleic acid hybridization for the differentiation of PRCV isolates from transmissible gastroenteritis virus (TGEV) isolates, and has yet to be submitted for publication. Patrick Halbur is listed as a secondary author on the manuscripts for providing clinical samples from which some of the PRCV isolates were obtained, and for providing his expert opinion as a pathologist in the determination of the pathogenicity of the PRCV isolates AR310, LEPP, and IA1894. An appendix describing the cloning of the TGEV S gene and expression of the S glycoprotein of TGEV in insect cells is also included and follows the Acknowledgments section of this work. In addition, a current review of the literature relevant to this dissertation is included as well as a General Conclusions section. Literature citations contained within the General Introduction, Literature Review, and Appendix are listed in the Literature Cited section.

Introduction

Porcine respiratory coronavirus (PRCV), a member of the family *Coronaviridae*, is antigenically related to transmissible gastroenteritis virus (TGEV) of swine. Coronaviruses are pleomorphic enveloped viruses with a positive-sense single stranded RNA genome (Spaan et al., 1988). Transmissible gastroenteritis virus causes severe diarrhea with a high mortality in neonatal swine as TGEV replicates in and destroys the enterocytes of the villus epithelium in the small intestine which causes the subsequent malabsorption and dehydration characteristic of transmissible gastroenteritis (TGE) (Saif and Bohl, 1986). Transmissible gastroenteritis virus has also been shown to replicate in respiratory tissue of infected swine (Laude et al., 1984; Underdahl et al., 1975). Porcine respiratory coronavirus is thought to be a mutant of TGEV as it is closely related to TGEV yet replicates to high titers in respiratory tissue with little or no clinical disease evident (Laude al., 1993; Pensaert et al., 1986). Porcine respiratory coronavirus appears to replicate very little, if at all, in intestinal tissue of infected swine (Cox et al., 1990a; Pensaert et al., 1986). Porcine respiratory coronavirus was first isolated in 1984 (Pensaert et al., 1986) from pigs in Belgium that were seropositive for TGEV but did not have a history of clinical TGE. Since the initial isolation of PRCV, infections of swine in Europe with PRCV are widespread (Cox et al., 1990a; Laude et al., 1993; Pensaert et al., 1986, Pensaert et al., 1993). Porcine respiratory coronavirus also has been found in the United States, but its prevalence in the United States is not known (Hill et al., 1990; Paul et al., 1992; Wesley et al., 1990b). Because TGEV and PRCV are closely related and yet display differences in tissue tropism and pathogenicity, they can serve as useful models for the study of coronavirus genes involved in tropism and virulence.

Porcine respiratory coronavirus is antigenically similar to TGEV in that polyclonal sera which neutralize TGEV also neutralize PRCV (Callebaut et al., 1989; Hill

et al., 1990; Pensaert et al., 1986; Wesley et al., 1990b). Anti-TGEV neutralizing monoclonal antibodies (MAbs) directed against the S glycoprotein readily neutralize PRCV; however, there are some non-neutralizing anti-TGEV MAbs directed against the S glycoprotein that can be used to distinguish between PRCV and TGEV isolates in a competitive binding assay (Callebaut et al., 1989; Garwes et al., 1988; Simkins et al., 1993; Van Nieuwstadt and Boonstra, 1992).

There are several similarities between PRCV and TGEV. Both viruses have three major structural proteins: the surface spike (S) glycoprotein, the integral membrane (M) glycoprotein, and an internal nucleoprotein (N). Nucleotide sequence of PRCV isolates thus far studied show that they are closely related to TGEV (Britton et al., 1991; Jackwood et al., 1992; Rasschaert et al., 1990; Wesley et al., 1991b). Previous studies of the genetic structure of PRCV have shown that all of the isolates have two unique characteristics. First, the S gene of PRCV contains a large inframe deletion ranging from 672- to 681-nucleotides in length (Britton et al., 1991; Jackwood et al., 1992; Rasschaert et al., 1990; Wesley et al., 1991b). This deletion in the S gene results in a smaller S glycoprotein. Second, all of the PRCVs analyzed thus far have had the CTAAAC consensus leader RNA binding site preceding the 3a gene altered or partially deleted (Page et al., 1991; Rasschaert et al., 1990; Wesley et al., 1991b). Thus, the subgenomic mRNA 3 is not detected in PRCV infected cells (Page et al., 1991; Wesley et al., 1991b). Also, the 3 gene of the European PRCVs contains several small deletions that render the 3 gene to a psuedogene (Page et al., 1991; Rasschaert et al., 1990). One PRCV isolate from the United States, Ind/89, was found to have an nearly intact 3 gene but the CTAAAC consensus leader RNA binding site had been altered to CTAAAT, thus making the subgenomic mRNA 3 of Ind/89 undetectable in infected cells (Wesley et al., 1991b). The presence of a large deletion in the 5' end of the S gene is thought to play a role in

determining the tissue tropism of PRCV (Parker et al., 1989; Sanchez et al., 1992). In the case of TGEV, the genes 3 and 3-1 (formerly designated as ORF A and ORF B, respectively) have been hypothesized to be important in the virulence and pathogenesis of TGEV infection (Wesley et al., 1991b). It is interesting to note that all previously described PRCV isolates have been demonstrated to have an altered or deleted 3 gene and that these PRCVs all cause minimal to no apparent clinical disease in swine.

My colleagues and I have been interested in obtaining isolates of PRCV to determine how prevalent this virus is in the swine population, to identify its role in pneumonia in swine, to better understand the mechanism of the origin of PRCV from TGEV, and to clarify the role of genes 3 and 3-1 in determining the pathogenicity of PRCV isolates. In the first study, my colleagues and I report on the isolation of three new PRCV isolates in the United States and present evidence that these isolates have deletions in the S gene that appear to be of varying sizes. Also, Halbur et al. (1994) recently have shown that three of the PRCV isolates analyzed in the study vary in pathogenicity. The PRCV isolates AR310 and LEPP caused moderate bronchointerstitial pneumonia in specific pathogen free (SPF) pigs, whereas the PRCV isolate IA1894 caused only a very mild bronchointerstitial pneumonia in five-week-old SPF pigs. This presented a unique opportunity to determine whether the S gene deletions and the 3 and 3-1 genes of these new PRCV isolates are different from previously studied isolates. If the 3 gene does play a role in the determination of pathogenicity, then the PRCV isolates AR310 and LEPP should have an intact 3 gene. Therefore, in the second study, my colleagues and I have analyzed the 5' end of the S gene and the 3 and 3-1 genes of four PRCV isolates. The size and location of the S gene deletion of the PRCV isolates was determined as my colleagues and I had previously shown that two of the PRCV isolates in this study, AR310 and LEPP, had smaller deletions in the 5' end of the S gene when

compared with another United States PRCV isolate designated ISU-1. Because the 3 and 3-1 genes have been hypothesized to determine the virulence of TGEV, my colleagues and I wished to examine the 3 and 3-1 genes of the two pathogenic and the one nonpathogenic PRCV isolates to determine if the 3 and 3-1 genes of these PRCV isolates could be related to the differences in virulence exhibited by the PRCV isolates. In the third study, my colleagues and I have used two cDNA probes from the S gene of TGEV that were developed in the first study to differentiate between PRCV and TGEV isolates. In this study, my colleagues and I used a nucleic acid hybridization assay that uses a immunochemiluminescent detection method to differentiate between TGEV and PRCV isolates, thus avoiding the use of radioactive materials usually used in a nucleic acid hybridization assay.

LITERATURE REVIEW

The History of Coronaviruses

The coronaviruses are large, enveloped, plus (+) stranded RNA viruses that belong to the *Coronaviridae* family of viruses and have been implicated in respiratory, cardiovascular, neurological, and enteric diseases of both humans and domestic animals (Holmes, 1990; Lai, 1990; Saif and Bohl, 1986). The coronaviruses are so named because of the petal-shaped glycoproteins projecting from the envelopes of virions that reveal a "corona" or crown-like appearance when viewed by electron microscopy (McIntosh, 1990). Schalk and Hawn are believed to be the first to describe a disease that was caused by a coronavirus when avian infectious bronchitis was differentiated from other respiratory ailments of chickens in 1931 (Schalk and Hawn, 1931). Later, TGE of swine was described in 1946 by Doyle and Hutchings, and murine hepatitis virus was recognized in 1949 by Cheever et al. In 1965, Tyrrell and Bynoe described recovering an ether-labile virus recovered from a boy with a cold and its subsequent passage in human embryonic tracheal organ cultures. This virus had a morphology that was very similar to that of avian infectious bronchitis virus when viewed by electron microscopy. McIntosh et al. (1967) had isolated other strains of human coronaviruses and showed that these human coronaviruses were related, both antigenically and morphologically, to murine hepatitis virus. At this point, a new genus was suggested for these viruses exhibiting a crown-like appearance (Tyrrell et al., 1968; Garwes et al., 1976). For many years, classification of coronaviruses was based solely on this characteristic morphology (Holmes 1990). However, coronaviruses can now be identified by properties of their structural proteins, genomic RNA and mRNAs, and by antigenic cross-reactivity and

nucleic acid homology with known coronaviruses, in addition to their morphology (Holmes, 1990).

Coronaviruses are divided into four antigenic groups, and generally infect only one species or several closely related species (Holmes, 1990). Table 1 shows the host species infected by coronaviruses.

TABLE 1. Coronaviruses: Antigenic groups, abbreviations, names, and hosts[a]

Antigenic group	Abbreviation	Name	Host
I	HCV-229E	Human respiratory coronavirus	Human
	TGEV	Transmissible gastro-enteritis virus	Pig
	PRCV	Porcine respiratory coronavirus	Pig
	CCV	Canine coronavirus	Dog
	FECV	Feline enteric coronavirus	Cat
	FIPV	Feline infectious peritonitis virus	Cat
II	HCV-OC43	Human respiratory coronavirus	Human
	MHV	Mouse hepatitis virus	Mouse
	HEV	Hemagglutinating encephalomyelitis virus	Pig
	BCV	Bovine coronavirus	Cow
	RbCV	Rabbit coronavirus	Rabbit
	SDAV	Sialodacryadentis virus	Rat
III	IBV	Infectious bronchitis virus	Chicken
IV	TCV	Turkey coronavirus	Turkey

[a] Adapted from Holmes (1990).

Coronaviruses

Examination of coronavirus particles by negative staining electron microscopy reveals that coronaviruses are generally spherical, yet pleomorphic, with a diameter ranging from 60 to 160 nanometers (nm) (Holmes, 1990; Saif and Bohl, 1986). The coronavirion is enveloped with a single layer of large, widely spaced club-shaped projections called peplomers (Holmes, 1990; Saif and Bohl, 1986). These peplomers evenly cover the virion surface and are 12 to 25 nm in length (Saif and Bohl, 1986). Coronavirus particles have a molecular mass of 6 to 9 x 10^6 and a buoyant density of 1.18 to 1.21 g/ml in sucrose and 1.14 to 1.23 g/ml in CsCl (Bohl, 1989; Brian et al., 1980; Garwes and Pocock, 1975).

Coronaviruses appear to use specific cellular receptors to infect specific host tissues via plasma membrane fusion. Blockage of the phagocytic or endosomal pathways by cytochalasin B or lysosomotropic bases did not affect coronavirus infection of susceptible cells (Kooi, et al., 1992; Payne et al., 1990). A receptor of 110 kDa has been found to be used by MHV to enter murine enterocytes and hepatic cells (Williams et al., 1990). This 110 kDa MHV receptor was found to be mmCGM1 (murine carcinoembryonic antigen gene family member 1) which belongs to the carcinoembryonic antigen family of proteins (Williams et al., 1991). Recently, mmCGM2 (murine carcinoembryonic antigen gene family member 2), another member of the carcinoembryonic antigen family, was found to be an alternative receptor for MHV (Yokomori and Lai, 1992). The receptor for both HCV-229E and TGEV has been found to be aminopeptidase N, a protein that is abundant in respiratory tissue and the intestinal tract (Delmas et al., 1992; Yeager et al., 1992).

The genomic RNA of coronaviruses is the largest among RNA viruses, approximately 27 to 32 kilobases (kb) in size (Spaan et al., 1988; Lai, 1990). When the

plus-strand genomic RNA of a coronavirus is extracted and introduced into a host cell, the genomic RNA is infectious and can serve directly as a mRNA template (Holmes, 1990; Spaan et al., 1988). The genomic RNA of coronaviruses is capped at its 5' end and is polyadenylated at its 3' end (Spaan et al., 1988). The replication of coronaviruses occurs exclusively in the cytoplasm of infected cells. Once the virus has penetrated the host cell, the genomic RNA attaches to ribosomes and a virus-specific RNA-dependent RNA polymerase is synthesized (Holmes, 1990). The polymerase regions of IBV and MHV has shown that in both cases "two ORFs are translated via a -1 frame shifting-pseudoknot" to produce the polymerase polyprotein. Several putative domains are found in the two polymerase ORFs. These domains include "membrane anchor, cysteine rich, and protease domains in ORF 1a" (Lee et al.; 1991, Olsen, 1993) and "polymerase, helicase, and zinc-finger domains and protease cleavage sites in ORF 1b" (Denison et al., 1991; Lee et al., 1991, Olsen, 1993). This RNA-dependent RNA polymerase activity transcribes the plus-strand genomic RNA into both full-length negative-strand RNA (Spaan et al., 1988) and subgenomic negative-strand RNAs (Lai, 1990). The negative-strand RNAs then serve as templates for the transcription of genomic RNA and subgenomic mRNAs (Spaan et al., 1988). The synthesis of coronavirus mRNAs occurs by leader primed transcription "in which a leader RNA is transcribed from one end of the RNA template, dissociates from the template, and then rejoins the template RNA at downstream transcription initiation sites to serve as a primer for transcription. This model is unlike any of the discontinuous transcription mechanisms known to exist" (Lai, 1990). Coronavirus "genomes contain approximately 220 to 500 nucleotides of untranslated sequence at the 5' end. The terminal 60 to 70 nucleotides are termed as the leader sequence and are transposed to the 5' ends of every mRNA species. This leader sequence is important in the regulation and transcription of coronavirus genomic RNA

and subgenomic mRNAs. At every intergenic region there is a small stretch of consensus sequence of approximately 8 to 10 nucleotides which is important for the downstream genes" (Lai, 1990). "The leader sequence of MHV is roughly 72 to 77 nucleotides long while that of IBV is roughly 60 nucleotides. A stretch of 7 to 18 nucleotides is homologous between the 3' end of the leader RNA and the intergenic regions. This homologous region is assumed to be the point of leader RNA binding and priming. Within this homologous region, seven nucleotides (UCUAAAC) are conserved among most coronaviruses. This conserved region is probably the consensus sequence recognized by RNA polymerases for mRNA transcription " (Lai, 1990). One nucleotide change in the consensus region "may account for the failure of a coronavirus to transcribe a mRNA. These results indicate the importance of both leader sequence and intergenic sequences in mRNA transcription " (Lai, 1990). At approximately 80 nucleotides from the 3' end of the genomic RNA, there is a stretch of sequences (CGAAGAGC) that is conserved in all the coronaviruses sequenced so far. This conserved sequence may be the recognition signal for the negative-stranded RNA synthesis" (Lai, 1990).

Once a susceptible cell is infected with a coronavirus, five to eight subgenomic mRNAs are generated, with the actual number varying among coronaviruses (Holmes, 1990; Spaan et al., 1988). The largest mRNA, designated number one, is identical in size to the virion genomic RNA (Lai, 1990). The subgenomic mRNAs form a coterminal 3' nested set in which they all have common 3' ends; thus, each mRNA contains all the nucleotide sequence in the next smaller mRNA plus one additional gene at the 5' end (Spaan et al., 1988; Holmes, 1990). This 5' end contains the ORF that is translated into the designated protein. "However, in vitro translation studies showed that only the 5' portion of each mRNA that does not overlap with the next smaller mRNA is translatable. Thus, these mRNAs are functionally monocistronic. Whether, the untranslated

downstream sequence in each mRNA serves any structural or functional role is not clear (Lai, 1990). However, some of the mRNAs of MHV, BCV, and IBV have recently been found to be bicistronic and tricistronic in nature (Boursnell et al., 1985; Liu et al., 1991; Liebowitz et al., 1985; Budzilowicz and Weiss, 1987; Senanayake et al., 1992). The predicted products from these bi- and tricistronic mRNAs have been detected in infected cells by using serum from animals naturally infected with the respective virus. Thus, the standard rule that only the 5' portion of each mRNA that does not overlap with the next smaller mRNA is translatable does not necessarily hold true. "The mRNAs are then used for the synthesis of structural and non-structural proteins. The functions of the non-structural proteins are not yet clear, some of them probably participate in viral RNA synthesis and others may be involved in virus assembly, and still others may be responsible for the shut-off of host macromolecular synthesis" (Lai, 1990).

Three major structural proteins are found in all coronaviruses, these are the nucleocapsid protein (N), the integral membrane glycoprotein (M), and the spike glycoprotein (S) (Spaan et al., 1988).

The peplomers are composed of the 180 to 200 kDa S glycoprotein. Only a small portion of the S glycoprotein is anchored in the lipid bilayer via a C-terminal domain, with the majority of the glycoprotein projecting to the outside surface of the virion in a coiled-coil structure (DeGroot et al., 1987; Spaan et al., 1988). The S glycoprotein is also involved in the attachment of the virion to specific cell receptors on the host cell, induction of cell-to-cell fusion, elicitation of neutralizing antibodies, and cell-mediated immunity (Holmes, 1990; Lai, 1990; Hogue et al., 1989). Most, but not all, of the neutralizing domains tend to be located in the amino terminal half of the S glycoprotein. The S glycoprotein is also thought to be involved in determining the tissue tropism of coronaviruses (Sanchez et al., 1992). The S glycoprotein of MHV has been

demonstrated to have a Fc-receptor-like activity (Oleszak et al., 1992). Like the HA protein of influenza viruses, the coronavirus S glycoprotein undergoes a trimerization to form a functional S glycoprotein via disulfide linkages between cysteine residues (Opstelten et al., 1993). The S glycoprotein is cotranslationally inserted through the rough endoplasmic reticulum (RER) membrane via a N-terminal signal peptide and undergoes N-linked glycosylation as the polypeptide grows in length (Holmes, 1990; Spaan et al., 1988). Most of the S glycoprotein synthesized in infected cells is incorporated into budding virions at pre-Golgi membranes with a smaller portion being transported to the plasma membrane (Holmes, 1990; Opstelten et al., 1993).

The N protein is a basic phosphoprotein with an apparent molecular mass ranging from 45 to 60 kDa that serves to bind the genomic RNA to form a helically symmetrical nucleocapsid (Holmes, 1990). The N protein appears to be an essential component in viral transcription as the level of N protein may act as the switch between transcription/mRNA synthesis and replication/genomic RNA synthesis (Lai, 1990, Olsen, 1993).

The M protein is a glycosylated transmembrane protein of an apparent molecular mass of 25 to 30 kDa that spans the host cell derived lipid bilayer several times (Holmes, 1990). The M protein of FIPV, FECV, and TGEV is N-linked; however, the M protein of MHV is O-linked (Spaan et al., 1988). Also the M protein serves as a base to which the nucleocapsid protein and its attached genomic RNA can anchor to the lumen of the lipid bilayer (Holmes, 1990). The M glycoprotein is also synthesized on membrane-bound polysomes, but its insertion and processing differ from the other viral glycoproteins (Holmes, 1990). The M glycoprotein lacks a N-terminal signal sequence, and yet is still inserted into the RER membrane (Spaan et al., 1988). This membrane insertion is dependent on internal signal recognition sites in the first or third hydrophobic

domains of the M glycoprotein (Holmes, 1990; Spaan et al., 1988; Britton et al., 1988b). A putative N-terminal signal peptide of 17 residues has been identified for the transmissible gastroenteritis virus M glycoprotein (Laude et al., 1987), thus, the lack of the signal sequence for insertion of the M glycoprotein may not be universal among all coronaviruses (Spaan et al., 1988). The glycosylation of the M glycoprotein occurs well after the protein is synthesized, unlike the S and hemagglutination-esterase (HE) glycoproteins that are glycosylated as they are synthesized (Holmes, 1990).

A small number of coronaviruses also possess the HE glycoprotein that is involved in hemagglutination (Holmes, 1990; Hogue et al., 1989; Spaan et al., 1988). Many coronaviruses, including the HCV-OC43 (human respiratory coronavirus), BCV (bovine coronavirus), HEV (hemagglutinating encephalomyelitis virus), TCV (turkey coronavirus), and several strains of MHV (mouse hepatitis virus) all contain the HE glycoprotein (Spaan et al., 1988; Holmes 1990). When present, the HE glycoprotein is also involved in virion attachment to the host cell, in addition to the S glycoprotein (Holmes, 1990). The many remaining coronaviruses that lack the HE glycoprotein neither hemagglutinate nor have esterase activity (Holmes, 1990). As with the S glycoprotein, the HE glycoprotein is cotranslationally inserted through the RER membrane via a N-terminal signal peptide and glycosylated as the polypeptide grows in length (Holmes, 1990; Spaan et al., 1988).

The N protein and several non-structural proteins are apparently made on the polysomes in the cytoplasmic matrix (Holmes, 1990). In contrast, the synthesis and processing of the M, S and HE (if present) glycoproteins occurs on the polysomes attached to the RER (Holmes, 1990).

The assembly of the virions occurs with budding at the membranes between the RER and the Golgi apparatus of the infected cell (Holmes, 1990). The N protein and

newly synthesized genomic RNA come together and form a helical nucleocapsid, and this nucleocapsid binds to the cytoplasmic surface of the RER and Golgi apparatus membranes. The mouse hepatitis virus does not package subgenomic mRNAs (Van der Most et al., 1991). In contrast, the subgenomic mRNAs of BCV and TGEV have been shown to be packaged into virions (Hofmann et al., 1990; Sethna et al., 1991). The packaging signal recognized by the N protein in MHV infected cells has been determined to be located in the 3' end of gene 1b (Makino et al., 1990). The packaging signal recognized by the N protein in TGEV and BCV infected cells is thought to be located in either the leader sequence or the 3' end of the genome since these regions are contained in both genomic RNA and subgenomic mRNAs, thus allowing both genomic RNA and subgenomic mRNAs to be packaged into virions.

It is believed that the cytoplasmic domain of the M glycoprotein inserted in the RER and Golgi apparatus membranes serves as an anchor for the binding of the nucleocapsid (Holmes, 1990). "This interaction could be the focal point for virus assembly because virus budding appears to occur at the site of the M accumulation" (Lai, 1990). The S glycoprotein is transported through the Golgi apparatus and is inserted into its membrane, where it may or may not be cleaved into two 90 kDa fragments (Holmes, 1990). Whether or not this cleavage of the S glycoprotein occurs depends on the particular coronavirus and the type of host cell infected. Excess S glycoprotein is transported to the plasma membrane of the host cell, and once at this location it will not be incorporated into any virions (Holmes, 1990). Opstelten et al. (1993) have observed that "the M protein associates with the S protein almost immediately after synthesis, forming large complexes which presumably accumulate at the site of budding and since budding occurs in the pre-Golgi membranes, this association is aimed at retaining the M protein and preventing it from escaping to the Golgi apparatus". Once a complete

nucleocapsid is bound to the M glycoprotein domain, a virion can then bud into the lumen of the RER and Golgi apparatus (Holmes, 1990). Virions containing a nucleocapsid, M, S, and HE (if present), are then released by cell lysis or by fusion of the Golgi apparatus derived, virion-containing, smooth-walled vesicles with the plasma membrane (Holmes, 1990). Since the M glycoprotein is restricted in its transport within the host cell (i.e., M can only be inserted in the cytoplasmic surface of the RER membrane), this accounts for the fact that coronaviruses can only bud from the RER and Golgi apparatus of the cell and not directly from the plasma membrane (Holmes, 1990).

The ability of coronaviruses to bud from the RER or the Golgi apparatus, and replication of viral subgenomic and genomic RNAs via 3' coterminal nested sets, are two very unique features of coronaviruses that enable them to be differentiated from other virus families.

The Transmissible Gastroenteritis Virus

In 1946, Doyle and Hutchings described a filterable agent that caused TGE in pigs. Transmissible gastroenteritis is a common and economically important disease of neonatal swine (Hill, 1989; Saif and Bohl, 1986). In Iowa alone, it is estimated that TGE cost the pork industry about $10 million annually in 1987 and 1988 (Hill, 1989). The extent that TGE will affect a swine herd depends on the immune status and age of the swine in the herd (Hill, 1989). The virus that is the etiologic agent of TGE is TGEV, and it is classified as a member of the *Coronaviridae* family of viruses.

Transmissible gastroenteritis is commonly divided into two major forms of the disease, these being the epizootic and enzootic forms (Saif and Bohl, 1986). The epizootic form of TGE occurs when the virus is introduced into a totally susceptible herd (Bohl, 1989), and is characterized by severe diarrhea, dehydration, vomiting, and high

mortality in young piglets (Saif and Bohl, 1986). The mortality in piglets under two weeks of age may reach 100%, but, in nursery age pigs, those of three to eight weeks of age, the mortality is usually less than 10 to 20% (Hill, 1989; Saif and Bohl, 1986). However, in these three- to eight-week-old piglets infected with TGE, the damage that has occurred to their small intestinal epithelium results in a slower and less efficient growth rate (Hill, 1989). In the older pigs in an affected herd, such as the growing and finishing pigs, sows, and boars, inappetence, vomiting, and diarrhea develops that lasts from two to four days (Hill, 1989; Saif and Bohl, 1986). Agalactia commonly occurs in lactating sows infected with TGEV, which in turn contributes to the severity of the disease in suckling pigs (Bohl, 1989). In the older age groups of pigs, the morbidity may reach 100% while the mortality is usually less than 5% (Hill, 1989). The epizootic form of TGE has traditionally been thought of as usually occurring in the winter months (Saif and Bohl, 1986), when the colder temperatures allow the labile TGEV to remain viable and be spread more readily.

The enzootic form of TGE is attributed to a continuance of the infection and the disease within a herd (Bohl, 1989). Weaned pigs are very susceptible to enzootic TGE since they have had a sudden loss of any passive immunity to TGEV that may have been passed on to them by suckling the sow (Hill, 1989; Bohl, 1989). Mortality is usually low, around 10 to 20% (Hill, 1989; Bohl, 1989), but, if enzootic TGE remains in a herd for over a year, the economic loses may exceed those incurred from the original epizootic outbreak (Hill, 1989). Generally, these losses are from a reduced growth rate and an increased susceptibility to other diseases (Hill, 1989).

There is an inverse relationship between the age of the animal infected with TGEV and the severity of the clinical signs, duration of the disease, and mortality (Bohl, 1989; Saif and Bohl, 1986). Pigs under two weeks of age will often have a mortality

rate of 100%. In most pigs under two weeks of age, death will occur in two to seven days after the onset of symptoms (Bohl, 1989). Three-week old or older swine show much less mortality, but they will have a decreased growth rate (Saif and Bohl, 1986). Transient vomiting, watery yellowish diarrhea, rapid weight loss, and dehydration are the major clinical signs of TGE (Bohl, 1989; Saif and Bohl, 1986). The affected young pigs will have profuse, foul-smelling diarrhea which often contains small curds of undigested milk (Saif and Bohl, 1986).

Growing, finishing, or adult swine have limited clinical signs associated with TGE. These signs include inappetence, a mild diarrhea of short duration, and occasional vomiting (Bohl, 1989; Saif and Bohl, 1986). Agalactia can also occur in lactating sows.

The site of virus replication in the small intestine is in the jejunum, and, to a lesser extent the ileum, with very little evidence of viral replication in the duodenum (Hooper and Haelterman, 1966). In the small intestine, a marked shortening or atrophy of the villi occurs and the walls of the small intestine become very thin (Hill, 1989; Garwes, 1988). The villi are shortened due to the loss and death of the villus enterocytes that are infected with TGEV (Pensaert et al., 1970). The crypt enterocytes do not appear to be infected, and as immature cells migrate up from the crypts to the tips of the villi to replace the infected villus enterocytes, they are more resistant to TGEV infection (Pensaert et al., 1970). The rate at which villus enterocytes can be replaced depends on the age of the pig. In normal one-day-old pigs, villus enterocytes can be replaced in seven to ten days, and in normal three-week-old pigs the villus enterocytes can be replaced in as little as two to four days (Moon, 1978). This difference in the time necessary to replace villus enterocytes can account for the higher mortality of newborn piglets infected with TGEV as compared to pigs of three weeks of age or older (Bohl, 1989).

There is evidence that TGEV can replicate in sites other than the gastrointestinal tract of swine. Transmissible gastroenteritis virus has been found in the milk of infected sows (Kemeny and Woods, 1977), in the respiratory tract of infected pigs (Underdahl et al., 1975). Also, TGEV also has been shown to replicate in alveolar macrophages (Laude et al., 1984).

Being a coronavirus, TGEV is enveloped and pleomorphic with characteristic club-shaped surface projections or peplomers. The nucleic acid of TGEV is single stranded RNA of positive polarity (Holmes, 1990; Spaan et al., 1988), and can be directly infectious (Norman et al., 1968). Transmissible gastroenteritis virus has three structural viral proteins, these being M, S, and N. The M protein is glycosylated with an apparent molecular mass of 25 to 30 kDa and is associated with the viral envelope. The S protein, has an apparent molecular mass of 200 kDa and is also glycosylated (Garwes et al., 1976). The N protein is not glycosylated as it is a basic phosphoprotein, has an apparent molecular mass of 45 to 50 kDa, is also associated with the binding of viral RNA, and serves as a nucleocapsid (Britton et al., 1988a). Antibodies directed against the S protein are neutralizing(Garwes et al., 1978/79). Transmissible gastroenteritis virus lacks a HE glycoprotein that is present in many other coronaviruses (Holmes, 1990).

Being an enveloped virus, TGEV is inactivated by ether and chloroform. Transmissible gastroenteritis virus is relatively stable at pH 3 and is trypsin resistant, as are enteric viruses in general (Saif and Bohl, 1986).

Transmissible gastroenteritis virus is routinely grown in the swine testicular cell line (McClurkin and Norman, 1966), in which TGEV shows a distinctive cytopathic effect (CPE). Through the years, several different cell lines were utilized in the attempt to propagate TGEV outside of the natural host. Only primary swine kidney cells (Harada

et al., 1963; Witte and Easterday, 1967), primary swine thyroid cells (Witte and Easterday, 1967; Dulac et al., 1977), and swine testicular cells (McClurkin and Norman, 1966) show a distinct CPE (Bohl, 1989).

Transmissible gastroenteritis virus is antigenically related to the feline infectious peritonitis virus (FIPV) and canine coronavirus (CCV), as antisera produced against these viruses react with TGEV (Horzinek et al., 1982; Sanchez et al., 1990). Porcine respiratory coronavirus has been shown to be closely related antigenically to TGEV, as antisera produced against both viruses react strongly with each of the viruses (Pensaert et al., 1986). However, the PRCV has a tropism for the respiratory tract of pigs and does not cause enteric disease symptoms (Cox et al., 1990a). Transmissible gastroenteritis virus shows no antigenic cross reactivity to the other porcine coronaviruses, namely, hemagglutinating encephalomyelitis virus (HEV) and porcine epidemic diarrhea virus (PEDV or CV777) (Pensaert et al., 1981; Egerbrink et al., 1988).

The replication of TGEV in a target cell line results in the synthesis of seven or eight mRNAs, depending on the TGEV strain. The full-length genomic mRNA 1 (approximately 25 kb) probably encodes for a RNA-dependent RNA polymerase. The S protein is encoded by the 8.2 kb subgenomic mRNA 2 (Wesley et al., 1991b). In the case of the virulent Miller strain of TGEV, the two nonstructural proteins, 3a and 3-1, are encoded by the 3.8 kb subgenomic mRNA 3 and by the 3.5 kb subgenomic mRNA 3-1, respectively (Wesley et al., 1989). The 3a and 3-1 regions are encoded by a single 3.8 kb subgenomic mRNA 3 in the virulent FS/72 British strain and the Purdue strain of TGEV (Britton et al., 1989; Kapke et al., 1988). The small integral membrane (sM) protein is associated with the viral envelope and is encoded by subgenomic mRNA 4 (Godet et al., 1992). The M and N proteins are encoded by the 2.5 kb subgenomic mRNA 5 and by the 1.7 kb subgenomic mRNA 6, respectively (Wesley et al., 1991b).

The 0.5 kb subgenomic mRNA 7 is the smallest subgenomic mRNA and has been shown to encode for a possible DNA binding protein in TGEV infected cells (Garwes et al., 1989; Britton et al., 1990). Preceding each large potential ORF is the conserved hexameric sequence of CTAAAC, which is thought to serve as a consensus leader RNA binding site in the transcription of the subgenomic mRNAs from a full-length negative sense template.

In the case of TGEV, the 3a and 3-1 genes (formerly designated as ORFs A and B, respectively) have been hypothesized to be important in the virulence and pathogenesis of TGEV infection (Wesley et al., 1990a). The small plaque (SP) mutant of TGEV was determined to have a S gene like that of TGEV, but that the entire 3a and 3-1 regions were missing due to a 462-nucleotide deletion (Wesley et al., 1990a). When three-day-old piglets were infected with SP virus, no signs of TGE were observed (Woods 1978). The SP mutant was found to replicate in the cells of the lamina propria rather than the villus enterocytes of the small intestine (Wesley et al., 1990a; Woods et al., 1981, Woods, 1978). Wesley et al. (1990a) concluded that the 3a and 3-1 genes are not needed for virus replication and hypothesized that the 3a and 3-1 genes "appear to be the contributing entities for TGEV virulence, SP morphology, tissue tropism, and/or persistence in swine leukocytes". The extent of SP virus replication in the respiratory tissue of infected swine has not been reported.

Until the polymerase region of the coronavirus genome from virulent and avirulent coronaviruses can be studied in depth, the area of the genome most likely to be involved in determining pathogenicity will be genes 3 and 3-1. No function has been assigned to the proteins encoded from the genes 3 and 3-1 as of yet.

The Porcine Respiratory Coronavirus Virus

Porcine respiratory coronavirus has been shown to be closely related to TGEV and yet replicates to high titers in respiratory tissue with little or no clinical disease evident. Porcine respiratory coronavirus appears to replicate very little, if at all, in intestinal tissue of infected swine. Porcine respiratory coronavirus is widespread in Europe where it was first found. Porcine respiratory coronavirus has also been found in the United States, but not to the extent that has been found in Europe.

Evidence for PRCV was first found in Belgium in 1984 when a routine serological survey of sows indicated that 68% of the sows had neutralizing antibodies to TGEV (Pensaert et al., 1986). This was a dramatic increase as the earlier surveys had shown that the percentage of sows with anti-TGEV antibodies to be 12 to 24%. (Pensaert et al., 1986). This dramatic increase in seropositive sows was even more confusing because the diarrhea typically associated with TGE was not evident in the seropositive herds. Further studies with sentinel pigs placed among these herds showed that the sentinel pigs seroconverted to TGEV but had not demonstrated any clinical signs typical of TGE. Eventually, a "TGE-like mutant" was isolated from nasal swabs and was designated TLM83. The isolate TLM83 was shown to be a coronavirus and cross reacted strongly with anti-TGEV serum. Later, the name TLM83 was changed to PRCV because of the almost exclusive respiratory tropism exhibited by this virus.

By utilizing a classical virus neutralization test, antibodies directed towards TGEV or PRCV can not be differentiated. Thus, determining whether swine had been previously infected with PRCV or TGEV became an important issue among swine producers as "the wide prevalence of PRCV had created serious obstacles for export of pigs to TGE-free countries" (Laude et al., 1993). Several differential competitive inhibition ELISA tests have been developed that can discern whether antibodies were

produced in response to TGEV or PRCV (Garwes et al., 1988; Callebaut et al., 1989; Van Nieuwstadt and Boonstra, 1992; Simkins et al., 1993). All of these ELISA procedures are developed on the same concept of using "a non-neutralizing MAb directed against an epitope on the S protein of TGEV with no counterpart in PRCV, in order to permit the detection of TGEV-specific antibodies without interference of PRCV antibodies" (Laude et al., 1993). Thus, it is the "combination of a negative result in the competitive blocking ELISA and a positive result in the seroneutralization test that gives evidence of a PRCV infection" (Laude et al., 1993).

The use of cDNA probes in a dot blot nucleic acid hybridization assay have also been employed to differentiate between TGEV and PRCV isolates grown in the laboratory (Bae et al., 1991; Jackwood et al., 1992; Wesley et al., 1991a). These assays utilize cDNA probes from the 5' end of the TGEV S gene of which there is no counterpart region found in known PRCV isolates.

Porcine respiratory coronavirus is readily adapted to growth in cell culture. The CPE induced by PRCV in susceptible cell cultures is often apparent on the first passage, compared to the 3 or 4 blind passages in cell culture needed before TGEV field strains induce CPE. Porcine respiratory coronavirus is are readily grown in swine testicle (ST) cells, swine kidney (PK15) cells, primary swine kidney cells, and a continuous cell line of cat fetuses (FCWF) (Laude et al., 1993). The ST cell line developed by McClurkin and Norman (1966) has been determined to be the most susceptible to PRCV infection (Pensaert, 1989).

The antigenic similarities between TGEV and PRCV, plus the fact the "wide distribution of PRCV in the European swine population, has been accompanied by a marked reduction in the number of TGEV outbreaks" (Laude et al., 1993), caused questions to arise about the possibility of using PRCV as a vaccine to induce lactogenic

immunity against TGE. As described previously, lactogenic immunity refers to the development of IgA anti-TGEV antibodies in sow's milk that can then be passed on to suckling piglets. After a natural PRCV infection, sows do secrete TGEV-neutralizing antibodies in their milk (Laude et al., 1993), though not always of the IgA class (Callebaut et al., 1990; Laude et al., 1993). Several researchers have used PRCV as a potential vaccine to induce lactogenic immunity against TGE and have obtained varying results. Paton and Brown (1990) found that following TGEV challenge, PRCV-vaccinated sows provided no passive protection to nursing piglets. Bernard et al. (1989) and De Diego et al. (1992) showed that 47% and 56% of piglets nursing on PRCV-vaccinated sows were protected from TGEV challenge, respectively. Recently, Wesley and Woods (1993) showed that 70% of piglets nursing on PRCV-vaccinated gilts were protected against TGEV challenge as compared with a 16% survival rate for TGEV-challenged piglets nursing on nonimmunized gilts. Wesley and Woods (1993) speculated that the higher level of protection achieved in their study was probably due to the fact that one of the immunizations was an intramuscular injection of PRCV emulsified in adjuvant, which resulted in higher virus neutralization titers in the vaccinated gilts serum. As for an explanation of the sharp reduction of TGEV outbreaks in Europe, Laude et al. (1993) propose that "regular PRCV-infection waves on breeding farms most probably favor and maintain the presence of anti-TGEV IgA in sow milk". Laude et al. (1993) further propose that "in PRCV-immune breeding herds, TGEV outbreaks are of a milder course which may be due to the presence of lactogenic IgA in sow milk, but they are also of a shorter duration". "When PRCV-primed sows subsequently become infected with TGEV, an amnestic response will quickly develop" and "a rapid increase of IgA antibodies in milk will occur and piglets born a few days after the start of the [TGEV] outbreak already receive a solid lactogenic immunity" (Laude et al., 1993).

The ability of PRCV-vaccinated swine to develop an intestinal immunity to TGEV is also unclear. Van Nieuwstatdt et al. (1989) could not demonstrate a reduction of TGEV antigen in the feces of PRCV-immune specific pathogen free (SPF) pigs. However, Cox et al. (1993) found that although "PRCV-immune pigs showed serological evidence of TGEV replication" the "PRCV-immune pigs eliminated TGEV infection more rapidly, or that they produced lower quantities of infectious virus than the seronegative pigs". Van Nieuwstatdt et al. (1989) utilized an ELISA that detected both infectious virus and inactivated virus, whereas Cox et al. (1993) used a competitive inhibition ELISA for the differentiation of serum antibodies directed against TGEV or PRCV and attempted to isolate infectious TGEV from experimentally challenged pigs. However, both of the studies by Van Nieuwstatdt et al. (1989) and Cox et al. (1993) indicated that when PRCV-immune pigs were challenged with TGEV a rapid secondary humoral immune response occurred.

The extent of clinical disease signs caused by natural PRCV infection are debatable. Originally, PRCV was thought to be non-pathogenic since the first described isolate TLM83 was isolated from clinically normal swine (Pensaert et al., 1986). The role of PRCV in respiratory disease is most likely affected by "multiple environmental factors such as climate, housing, and concomitant infections" (Laude et al., 1993). As an example, two separate experiments gave conflicting information as to the pathogenicity of PRCV when combined with swine influenza in experimentally infected swine. Lanza et al. (1992) showed that dual infection with PRCV and swine influenza did not appear to affect clinical signs and lung lesions. However, Laude et al. (1993) described unpublished data that indicated that "clinical signs and lung lesions were more severe in dual infected animals than in those infected with either of the respiratory agents alone".

The occurrence of clinical disease and lung lesion development in swine experimentally infected with PRCV also varies. O'Toole et al. (1989) described one-week-old SPF pigs oronasally infected with a British PRCV isolate designated as Stopps, to be clinically healthy, have no clinical disease, but had mild bronchointerstitial pneumonia upon histological examination. Cox et al. (1990a) infected one-week-old SPF pigs by aerosol with TLM83 and reported a "diffuse interstitial pneumonia" but that the pigs also experienced no respiratory distress. Five-week-old SPF pigs inoculated oronasally with a PRCV isolate from the Netherlands showed no clinical signs of respiratory disease but did display a catarrhal bronchopneumonia at necropsy (Van Nieuwstatdt and Pol, 1989). Vannier (1990) utilized intratracheal inoculation to infect 90 day-old SPF pigs and produced mild clinical signs of dyspnea, polypnea, and a short lasting fever. The United States PRCV isolate Ind/89 was inoculated into conventional and hysterectomy-derived colostrum-deprived (HDCD) neonatal piglets and no clinical disease was observed (Wesley et al., 1990b). Another United States PRCV isolate designated ISU-1 failed to induce clinical disease in experimentally infected HDCD swine (Hill et al., 1989). Laude et al. (1993) described unpublished data from experiments using "fattening pigs" that were inoculated by aerosol with 7 Belgian PRCV isolates. In this experiment, Laude et al. (1993) described the development of "mild respiratory signs such as nasal discharge and sneezing", and that "variation in virulence between the isolates was not detected". Halbur et al. (1993) intranasally inoculated three-day-old gnotobiotic pigs with a new United States PRCV isolate designated AR310, and microscopic examination of lung tissue revealed a "necrotizing and proliferative bronchointerstitial pneumonia characterized by necrosis, squamous metaplasia, dysplasia, proliferation of airway epithelium, mononuclear cell infiltration of alveolar septa, mild type II pneumocyte proliferation, and lymphohistiocytic exudation". The

lesions described by Halbur et al. (1993) were "mild by 3 days post inoculation (DPI), moderate by 5 DPI, severe by 10 DPI, and mostly resolved by 15 DPI" with no clinical disease observed among the infected gnotobiotic pigs. Halbur et al. (1993) speculated that lack of clinical disease in the PRCV AR310 infected gnotobiotic pigs was due to the gnotobiotic environment, and hypothesized that if PRCV AR310 " causes similar lesions in conventional pigs, it might predispose them to secondary bacterial infections". Halbur et al. (1994) has shown that when PRCV AR310 was intranasally inoculated into five-week-old SPF pigs the results showed that PRCV AR310 caused moderate bronchointerstitial pneumonia. In contrast, when another PRCV isolate designated IA1894 was inoculated into five-week-old SPF pigs in the same experiment the results showed that PRCV IA1894 caused only mild bronchointerstitial pneumonia. Laude et al. (1993) proposed that the diversity in presentation of clinical disease signs among experimentally PRCV infected swine "may be influenced by the age of pigs and/or the inoculation technique". However, the recent findings by Halbur et al. (1994) indicate that the pathogenicity of some PRCV isolates in five-week-old SPF pigs may vary based on clinical disease signs and lung lesion development. More study into the role of coronavirus genes involved in tissue tropism, pathogenesis, and virulence should help clarify why some recent PRCV isolates vary in clinical disease signs and lung lesion development.

Porcine respiratory coronavirus has been found to replicate in a variety of anatomical sites in swine. In experimentally infected swine, PRCV has been easily isolated from lung, trachea, nasal mucosa, tonsils, stomach, and small intestine and its contents (O'Toole et al., 1989; Cox et al., 1990a). Porcine respiratory coronavirus has been occasionally isolated from lymph nodes, thymus, spleen, liver, kidney, and leukocytes (Cox et al., 1990a). The replication of PRCV in the gastrointestinal tract is

limited and does not destroy villus enterocytes as does TGEV. By using immunofluorescent detection, the cells in the intestinal tract from PRCV infected swine containing viral antigen were villus enterocytes, crypt cells, and unidentifiable cells under the villus enterocytes and crypts (Cox et al., 1990a). Viremia and/or ingestion of PRCV produced in the respiratory tissue were proposed mechanisms for the ability of PRCV to get to the intestinal tract and undergo limited replication (Cox et al., 1990a). The limited ability of PRCV to replicate in the intestine was quantified by Cox et al. (1990b) as it took over 10^3 $TCID_{50}$ of PRCV directly inoculated into the intestines of swine to start intestinal infection. Although European researchers have routinely isolated PRCV from the intestines of experimentally infected swine (O'Toole et al., 1989; Cox et al., 1990a; Cox et al., 1990b), researchers in the United States have not isolated PRCV from intestinal homogenates from experimentally infected swine (Halbur et al., 1993; Hill et al., 1989; Wesley et al., 1990). United States researchers have isolated PRCV from rectal swabs collected from experimentally infected pigs which may reflect the ingestion of PRCV produced in respiratory tissue (Halbur et al., 1993; Wesley et al., 1990b).

The sudden occurrence of PRCV was in itself puzzling, but an even more intriguing puzzle was determining the origin of PRCV. It was suggested that PRCV might actually be a TGEV vaccine that had developed an extreme tropism for respiratory tissue. Other hypothesis considered the possibility that PRCV was a result of "laboratory manipulation" or the "spontaneous genome recombination between TGEV and another heterologous coronavirus" (Laude et al, 1993). But information provided by the nucleotide sequence analysis of PRCV isolates shows that PRCV most likely arose from TGEV itself. Nucleotide sequence analysis thus far has shown that PRCV isolates have no sequences unique to themselves when compared to the genome of TGEV, however, PRCV isolates do demonstrate some consistent nucleotide deletions.

There are several similarities between PRCV and TGEV. Both viruses have three major structural proteins: the surface S glycoprotein, the M glycoprotein, and an internal N protein. Previous studies of the genetic structure of PRCV have shown that the isolates all have had two unique characteristics. First, the S gene of PRCV contains a large inframe deletion ranging from 672- to 681-nucleotides in length (Rasschaert et al., 1990; Britton et al., 1991; Wesley et al., 1991b; Jackwood et al., 1992). This deletion in the S gene results in a smaller S glycoprotein. Second, all of the PRCVs analyzed thus far have had the CTAAAC consensus leader RNA binding site preceding the 3a gene altered or partially deleted (Rasschaert et al., 1990; Britton et al., 1991). Also, the 3a gene of the European PRCVs contains several small deletions that render the 3a gene to a psuedogene. One PRCV isolate from the United States, Ind/89, was found to have an intact 3a gene but the CTAAAC consensus leader RNA binding site had been altered to CTAAAT, thus making the subgenomic mRNA 3 of Ind/89 undetectable in infected cells (Wesley et al., 1991b).

The presence of a large deletion in the 5' end of the S gene is thought to play a role in determining the tissue tropism of PRCV. Sanchez et al. (1992) have proposed that four amino acid residues (92, 94, 218, and 219) in the S protein of TGEV are important in the binding of virus to enterocytes and that the tropism of certain strains of TGEV may be shifted from the intestinal tract to the respiratory tract. The amino acid residues 92, 94, 218, and 219 are within the region of the S gene that is characteristically deleted in PRCV isolates. Studies with the MHV coronavirus have shown that small deletions or point mutations within the 5' end of the S gene results in reduced neurovirulence (Dalziel et al., 1986; Fleming et al., 1986) However, it should be pointed out that these MHV studies have looked exclusively at the S gene of MHV mutants and

have not taken into consideration any other possible changes in the genome of the attenuated MHV mutants.

A protein that is expressed on the brush border membrane of small intestinal villi, designated aminopeptidase N (APN), has been shown to serve as a receptor for TGEV (Delmas et al., 1992). Aminopeptidase N also is "most likely to be expressed on respiratory epithelial cells and alveolar macrophages" (Laude et al., 1993) and PRCV may also use APN as a receptor to gain entry into cells. The mmCGM1 (murine carcinoembryonic antigen gene family member 1) was the first protein to be shown to serve as a receptor for MHV, however, an alternate receptor mmCGM2 (murine carcinoembryonic antigen gene family member 2) has been shown to be used by MHV to gain entry into cells (Williams et al., 1991; Yokomori and Lai, 1992). Thus, there is a possibility that PRCV may use an alternative receptor other than APN to gain entry into cells.

Laude et al. (1993) presents two possible explanations on why PRCV replicates very little if at all in the intestinal tissue of pigs. First, Laude et al. (1993) proposes that the "N-terminal domain of the PRCV S protein contributes in some way to the stability" of the whole S protein. Thus the "physiochemical environment of the digestive tract could result in a decreased attachment or fusogenic capacity of the PRCV S protein" if indeed it was destabilized due to alteration of the N-terminal domain" (Laude et al., 1993). Second, Laude et al. (1993) also consider the possibility that "PRCV restriction may take place after penetration into the enterocyte" and that this focuses "attention towards the second alteration of the PRCV genome, i.e. the conversion of the 3 gene to a pseudogene".

In the case of TGEV, the 3 and 3-1 genes (formerly designated as ORFs A and B, respectively) have been hypothesized to be important in the virulence and

pathogenicity of TGEV (Wesley et al., 1990a). It is interesting to note that all previously described PRCV isolates have been demonstrated to have an altered or deleted 3 gene and that these PRCVs all cause no apparent disease in swine. Wesley et al. (1990a) have hypothesized that the role of the 3 and 3-1 regions of TGEV are involved in pathogenesis and virulence. This hypothesis can be extended to PRCV.

In this study, my colleagues and I have analyzed the 5' end of the S gene and the 3 and 3-1 genes of four PRCV isolates. My colleagues and I wished to determine the size and location of the S gene deletion of each of the PRCV isolates. Also, Halbur et al. (1994) has recently shown that three of the PRCV isolates analyzed in this study vary in pathogenicity. The PRCV isolates AR310 and LEPP caused moderate bronchointerstitial pneumonia in SPF pigs, whereas the PRCV isolate IA1894 caused only a very mild bronchointerstitial pneumonia in SPF pigs. Since the 3 and 3-1 genes have been hypothesized to determine the virulence of TGEV, my colleagues and I wished to examine the 3 and 3-1 genes of the two pathogenic and the one nonpathogenic PRCV isolates to determine if the 3 and 3-1 genes of these PRCV isolates could be related to the differences in virulence exhibited by the PRCV isolates.

THREE NEW ISOLATES OF PORCINE RESPIRATORY CORONAVIRUS WITH VARYING PATHOGENICITY AND S GENE DELETIONS

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Abstract

Three new isolates of porcine respiratory coronavirus (PRCV) were isolated and partially characterized. These PRCV isolates showed a selective tropism for respiratory tissue and were antigenically related to transmissible gastroenteritis virus (TGEV). Polymerase chain reaction (PCR) amplification of the 5'-half of the spike (S) genes of the three PRCV isolates indicated that a large deletion, characteristic of PRCV, was present. Using cDNA probes specific for the TGEV S gene, the PCR products were shown to be specific in a Southern blot. The three new PRCV isolates were shown to vary in S gene deletion size. In a separate study, these isolates have also been shown to vary in pathogenicity. These new PRCV isolates should serve as important tools in gaining a better understanding of the pathogenesis of coronavirus infections.

Note

Porcine respiratory coronavirus (PRCV), a member of the family *Coronaviridae*, is antigenically related to transmissible gastroenteritis virus (TGEV) of swine. Porcine respiratory coronavirus was first isolated in 1984 (14) from pigs in Belgium that were seropositive for TGEV but did not have a history of clinical transmissible gastroenteritis (TGE). Since the initial isolation of PRCV, infections of swine in Europe with PRCV

are widespread (13,19). Porcine respiratory coronavirus has also been isolated from three herds in the United States (7,12,23). The prevalence of PRCV infections in United States swine is currently not known, as several investigators in the United States have been attempting to isolate PRCV without much success.

There are several similarities between PRCV and TGEV. Both viruses have three major structural proteins: the surface spike (S) glycoprotein, the integral membrane (M) glycoprotein, and an internal nucleoprotein (N). Nucleotide sequence of PRCV isolates thus far studied show that they are closely related to TGEV (1,8,15,21). However, there are some striking differences in that PRCV isolates have a deletion in the 5' end of the S gene (Figure 1); also, there are deletions in the nonstructural region coded by mRNA 3, when compared to TGEV (1,15). Porcine respiratory coronavirus has a different tissue tropism than TGEV. TGEV replicates in both the respiratory and intestinal tissues and causes gastroenteritis, whereas PRCV replicates to high titers in lung tissue of swine and with little or no replication in the intestinal tissues and no evidence of villus atrophy and gastroenteritis (3,4,10). Currently, the presence of the deletion in the S gene is thought to contribute to the differences in tissue tropism shown by these viruses (9,11,17). The nonstructural genes encoded by mRNA 3 and 3-1 are hypothesized to be involved in the pathogenesis of TGEV (9,21,22).

Porcine respiratory coronavirus is antigenically similar to TGEV in that polyclonal sera which neutralizes TGEV also neutralizes PRCV (2,7,23). Anti-TGEV neutralizing monoclonal antibodies (MAbs) directed against the S glycoprotein readily neutralize PRCV, however, there are some non-neutralizing anti-TGEV MAbs directed against the S glycoprotein that can be used to distinguish between PRCV and TGEV isolates in a competitive binding assay (2,5,18,19).

Of the European PRCV isolates that have had their nucleotide sequences published all have a 672-nucleotide deletion in the 5' end of the S gene (1,15,17). The U.S. PRCV isolates Ind/89 and ISU-1 had a 681-nucleotide deletion present in the 5' end of the S gene (8,21). This deletion in the 5' end of the PRCV S gene yields a smaller S glycoprotein.

My colleagues and I have been interested in obtaining additional isolates of PRCV in order to determine how prevalent this virus is in the swine population, to identify its role in pneumonia in swine, and to better understand the mechanism of the origin of PRCV from TGEV. Here, my colleagues and I report on the isolation of three new PRCV isolates in the United States and present evidence that these isolates have deletions in the S gene that appear to be of varying sizes.

The PRCV isolate AR310 was isolated from the small intestine of a pig from an Arkansas swine herd which suffered from endemic TGE. Approximately 10 percent suspensions of the intestinal sample were prepared in 0.05 *M* phosphate buffered saline (pH 7.4) and clarified by centrifugation at 1000 x g for 10 minutes. The supernatants were harvested, passed through a 0.45 μm filter (Costar, Cambridge, MA) and stored at -70°C until viral isolation was attempted. The swine testicular (ST) cell line was used to isolate and propagate the viruses in this study and were maintained as previously described (23). Four-day-old ST cells in 25 cm^2 flasks (Costar, Cambridge, MA) were treated with minimum essential medium (MEM) containing DEAE-dextran (50 μg/ml) (Sigma, St. Louis, MO) at 37°C for 30 minutes before the intestinal tissue filtrate was added. Prior to inoculation onto ST cell monolayers, 0.2 ml of the intestinal filtrate was mixed with 0.8 ml of a 1:10 dilution of porcine anti-enterovirus (Group 8C) hyperimmune serum (virus neutralization titer of 1:1600) in MEM with 2 percent fetal bovine serum (FBS) and antibiotics (penicillin 20,000 U/ml, streptomycin 20,000 μg/ml,

and amphotericin B 50 μg/ml) (GIBCO BRL, Grand Island, NY), and were incubated at 37°C for one hour. The ST cell monolayers were inoculated with the entire 1 ml of virus and anti-enterovirus hyperimmune serum mixture, and the inoculum was adsorbed onto the ST cells for 60 minutes at 37°C, after which additional MEM with 2 percent FBS plus antibiotics was added. The cultures were incubated at 37°C for 48 hours and observed daily for cytopathic effect (CPE). After 48 hours, whether or not CPE was evident, all cultures were frozen and thawed three times, clarified by centrifugation at 1000 x g for 10 minutes, and used as inoculum for the next passage. Cytopathic effect was evident at the third passage of AR310 in ST cells.

Two of the PRCV isolates in this study, IA1894 and LEPP, were isolated from nasal swabs from swine from herds that had antibodies to TGEV but had not presented evidence of diarrhea, and thus were suspected of having a PRCV infection. Nasal swabs were collected and placed into one ml of MEM with 2 percent FBS and antibiotics and mixed on a vortex mixer for 20 seconds. Two hundred μl of the MEM was inoculated onto ST cells. Cytopathic effect was present on the first passage in ST cells for both IA1894 and LEPP. All three isolates were plaque purified a total of three times and stock virus was stored at -70°C.

The gut-passaged virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, IA), also referred to as CHV TGEV, was used as a standard TGEV strain in this study. The PRCV isolate ISU-1 was received as a plaque-purified preparation and was kindly provided by Dr. Howard Hill (Iowa State University Veterinary Diagnostic Laboratory) (7).

The PRCV isolates produced a CPE in ST cells similar to that of TGEV however syncytia formation was detected in the PRCV-infected ST cells that was not present in TGEV-infected ST cells. Both swine polyclonal anti-TGEV serum and the neutralizing

anti-TGEV S monoclonal antibody (MAb) MH11 (24) recognized the PRCV isolates AR310, LEPP, and IA1894 in an indirect immunofluorescence assay (Figure 2) performed as previously described (24).

Polymerase chain reaction (PCR) amplification of the 5'-half of the S genes of the three PRCV isolates was performed to determine if a large deletion, characteristic of PRCV, was present. ST cells were infected at a multiplicity of infection of approximately 0.1 PFU/cell with CHV TGEV, AR310, ISU-1, IA1894, or LEPP. At 19 hours post infection, the medium was removed and the total RNA was isolated from the infected ST cell monolayers by a rapid guanidinium thiocyanate method (Stratagene, La Jolla, CA). The RNA was washed with 70% ethanol and dissolved in DEPC-treated distilled water and stored at -70°C. First strand cDNA was made by avian myeloblastosis virus reverse transcriptase by using random oligonucleotide primers (Invitrogen, San Diego, CA). The cDNA-RNA hybrids were amplified by PCR using Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) by using specific primers (Table 1). The 5'-half of the S genes of TGEV and the PRCV isolates were amplified with the primers 21209 and 060704 under the following parameters: 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 5 minutes at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C; followed by 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 5 minutes at 72°C in a DNA thermal cycler (Coy Corporation, Grass Lake, MI).

The TGEV and PRCV S gene PCR products were separated on a 2% NuSieve GTG (FMC Bioproducts, Rockland, ME) agarose gel and then purified from the agarose gel by the Magic PCR prep method (Promega, Madison, WI). The purified PCR products were then separated on a 1.5% SeaKem ME (FMC Bioproducts, Rockland, ME) agarose gel (Figure 3). The PCR products of the PRCV isolates showed that there

was a deletion in the S gene in comparison to TGEV. The PCR products from the isolates AR310 and LEPP appeared to migrate at the same rate, whereas the PCR products from the isolates IA1894 and ISU-1 migrated more slowly. To confirm that the amplified PCR products were specific for the S gene, a Southern blot was performed (16). The PCR products were separated on a 1.5% SeaKem ME agarose gel, depurinated with 0.25 *N* HCl, denatured with 0.5 *N* NaOH and 1.5 *M* NaCl, neutralized with 0.1 *M* Tris-HCl (pH 7.5) and 1.5 *M* NaCl and then transferred by using a Posiblot pressure blotter (Stratagene, La Jolla, CA) with 10x SSC (1x SSC is 0.15 *M* NaCl plus 15 m*M* sodium citrate) to nylon membranes (Magna NT, Micron Separations Inc., Westboro, MA). After the transfer was complete, the membranes were baked at 80°C for 2 hours to fix the DNA. The membranes were prehybridized for 2 hours in a solution containing 50% formamide, 5x SSPE (1x SSPE is 0.18 *M* NaCl, 10 m*M* sodium phosphate, and 1 m*M* EDTA), 4x Denhardt's solution, 0.3% sodium dodecyl sulfate (SDS), and sonicated salmon sperm DNA (30 μg/ml) at 42°C. The cDNA probes used in the hybridization procedure were designated FP2 and FP1 (Figure 1) . FP2 is a 2.2 kb PCR product that was amplified by the primers 101004 and 060704 and cut with the *Bam*HI restriction enzyme and cloned in the phagemid vector pKS+ (Stratagene, La Jolla, CA). FP1 is a 0.6 kb PCR product that was amplified by the primers 101004 and 100907 and cut with *Bam*HI and *Eco*RI restriction enzymes and cloned in the phagemid vector pKS+. FP2 encompasses the 5'-half of the TGEV S gene and thus will hybridize to both TGEV and PRCV S genes. However, FP1 encompasses the region of the TGEV S gene that is deleted from all PRCV isolates known thus far and will only hybridize with the S gene of TGEV. Twenty-five ng each of FP1 and FP2 were labeled with [α ^{32}P]dCTP (ICN Biochemicals, Costa Mesa, CA) in the presence of random hexamer primers and Klenow fragment of DNA polymerase I (Amersham Corporation, Arlington

Heights, IL). Unincorporated [α ^{32}P]dCTP was removed by passing the labeled DNA through a Sephadex G-50 column (Boehringer Mannheim, Indianapolis, IN). The labeled FP1 and FP2 probes were then heated to 100°C, cooled on ice, and then added to the appropriate prehybridization reaction and allowed to hybridize overnight. The membranes were washed once in 2xSSC-0.3% SDS at room temperature, twice in 2xSSC-0.3% SDS at 65°C, and once with 0.2x SSC at room temperature. Autoradiographs were made by exposing X-ray film (RX film, Fuji Photo Film Co., Japan) to the membranes (Figure 4). As expected, the probe FP2 detected the PCR products of all the PRCV isolates and TGEV, whereas the probe FP1 detected only the TGEV PCR product. Thus the Southern blot reveals that the amplified PCR products from TGEV and the PRCV isolates were specific and that these new PRCV isolates have deletions in their S genes typical of PRCV yet the differences in the migration rate of the PRCV PCR products in an agarose gel indicates that the S genes of these PRCV isolates have deletions of varying sizes.

The PRCV isolate AR310 has been shown to cause interstitial pneumonia in gnotobiotic pigs (6). By using five-week-old SPF pigs, PRCV isolates AR310, LEPP, and IA1894 were shown to exhibit a tropism for respiratory tissue with no detectable replication in intestinal tissue (Halbur et al., 1994). Also, the pathogenicity of the PRCV isolates AR310, LEPP, and IA1894 was shown to vary. The PRCV isolates AR310 and LEPP induced moderate bronchointerstitial pneumonia in five-week-old SPF pigs, whereas the PRCV isolate IA1894 induced very mild bronchointerstitial pneumonia (Halbur et al., 1994). This information is summarized in Table 2.

Porcine respiratory coronavirus is believed to have originated from TGEV. Although the mechanism of the deletion occurring in the S gene of PRCV is not known, RNA recombination with polymerase jumping is a possible mechanism (9,17). Whether

immune pressure plays a role in the deletion of the S gene in PRCV is not known. The PRCV isolate AR310 is the first PRCV strain to be isolated from intestinal tissue. The herd in which AR310 was isolated had been vaccinated with a commercial TGEV vaccine and immune pressure may have played a role in the development of the S gene deletion of AR310. However, it should be noted that TGEV was also isolated from other pigs in the same herd from which AR310 was isolated. Whether the original AR310 intestinal homogenate contained a mixture of TGEV variants, or the S gene deletion of AR310 occurred during cell culture adaptation is under study at this time. Porcine respiratory coronavirus can cause a viremia (3) and a possible explanation for the isolation of PRCV AR310 from intestinal tissue homogenates may have been due to a PRCV viremia at the time tissue collection.

The RM4, 86/137004, TLM83, Ind/89 and ISU-1 PRCV isolates were isolated from clinically normal swine herds that had serum titers to TGEV yet had not presented signs of enteric disease associated with TGEV infection (1,7,13,15,23). One of the PRCV isolates in this study, AR310, was isolated from intestinal tissue of a piglet with TGE and is the first to be isolated from such conditions. The other PRCV isolates LEPP and IA1894 originated from pigs with pneumonia.

The size of the S gene from these PRCV isolates also varies. The deletion in the PCR product of AR310 is smaller than that of ISU-1 and has been determined to be 621-nucleotides (12). The size of the deletion present in the LEPP S gene appears to be similar to that of AR310. The size of the deletion of IA1894 appears to be similar to the 681-nucleotide deletion of ISU-1. Studies involving the cloning and sequencing of the S gene of these isolates are underway to determine the precise location and size of the deletion. The nonstructural regions of the PRCV isolates AR310, LEPP, and IA1894 are currently under study as it has been proposed that the genes 3 and 3-1 are important in

the pathogenesis of TGEV and may play a similar role in the pathogenicity of PRCV (9,21,22). The analysis of these nonstructural regions of these PRCV isolates should be of interest since these isolates do vary in pneumopathogenicity.

Acknowledgments

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TABLE 1. TGEV S gene specific oligonucleotide primers used in PCR to amplify regions of the S gene of PRCV and TGEV

Primer			
Name		Sequence	Nucleotide localization
021209	5'	gggaattcgGGGTAAGTTGCTCATTAGAAA 3'	56-76 [a]
060704	5'	ggggatccGCAGTGCCACGAGTCCTATCAT 3'	2462-2483 [a]
101004	5'	gggggatccAGAACTATAGGTAACCATTGG 3'	1678-1698 [b]
100907	5'	gggggaaTTCTAATGTAGTCGCACGCAT 3'	2230-2250 [b]

[a] From the sequence of D. Rasschaert and H. Laude. J. Gen. Virol. (1987) 68:1883-1890.

[b] From the sequence of P. Britton and K.W. Page. Virus Res. (1990) 18:71-80.

TABLE 2. Tissue tropism and pathogenicity of PRCV isolates AR310, LEPP, and IA1894

	Virus Replication [a]		Lesions [b]	
Isolate	Lung	Intestine	Lung	Intestine
AR310	+	-	+	-
LEPP	+	-	+	-
IA1894	-	-	±	-
Control	-	-	-	-

a Presence (+), or absence (-) of CPE after ST cells were infected with twenty percent lung or intestinal homogenates from five-week-old SPF pigs inoculated intranasally with 10^7 pfu of the appropriate virus.

b Lung lesions were determined by the presence of moderate (+), mild (±), or absence (-) of bronchointerstitial pneumonia. Intestinal lesions were determined by the presence (+), or absence (-) of villus atrophy.

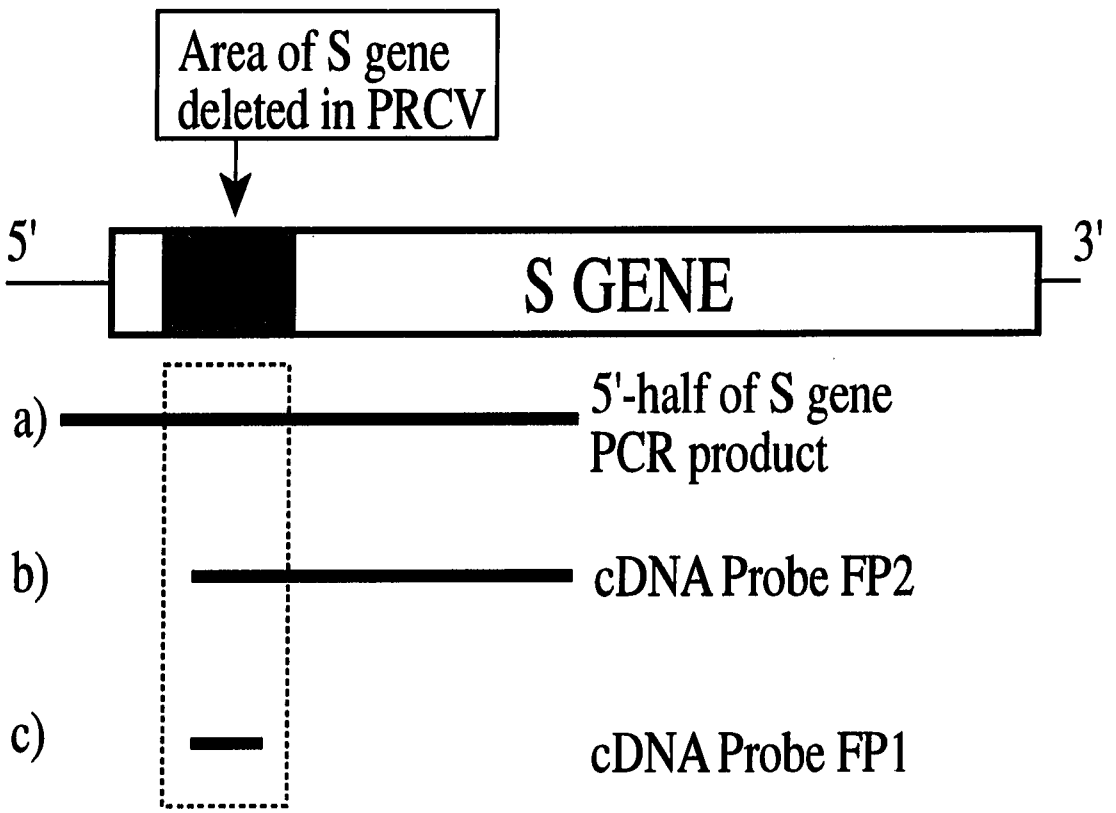


Figure 1. Schematic diagram of the S gene of TGEV with the characteristic deletion of PRCV shown. a) PCR product of the 5'-half of the S gene of TGEV and PRCV amplified by primers 021209 and 060704. b) Probe FP2 used to detect the S gene of both TGEV and PRCV in Southern blot. FP2 was amplified by the primers 101004 and 060704 and cloned in pKS+. c) Probe FP1 used to detect the S gene of TGEV in Southern blot. FP1 was amplified by the primers 101004 and 100907 and cloned in pKS+. Dashed line represents the area that is deleted in PRCV.

Figure 2. Immunofluorescent staining of PRCV AR310 infected (A), or uninfected (B), methanol/acetone fixed ST cells with the anti-TGEV S glycoprotein MAb MH11. The Miller strain of TGEV and the PRCV isolates LEPP, IA1894, and ISU-1 all reacted with the MAb MH11 in a similar pattern.

A

B

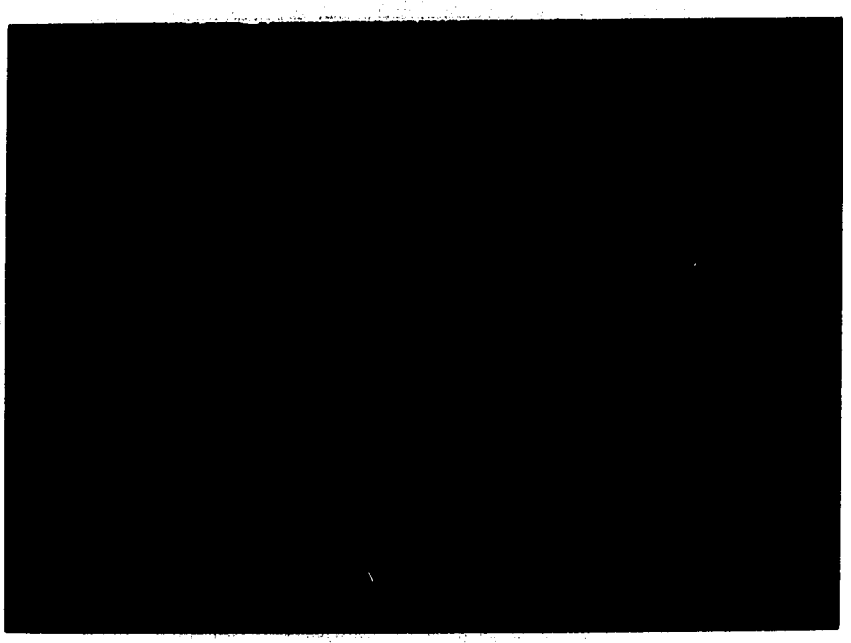


Figure 3. PCR amplification of the 5'-half of the S gene of TGEV and PRCV isolates. cDNA was amplified by PCR as with primers 021209 and 060704 as described in text and separated on 1.5% agarose gel. Molecular weight markers (M), CHV TGEV (lane 1), ISU-1 (lane 2), AR310 (lane 3), IA1894 (lane 4), LEPP (lane 5). Note that the PCR products from AR310 and LEPP migrate at the same rate, whereas the PCR products from ISU-1 and IA1894 migrate more slowly.

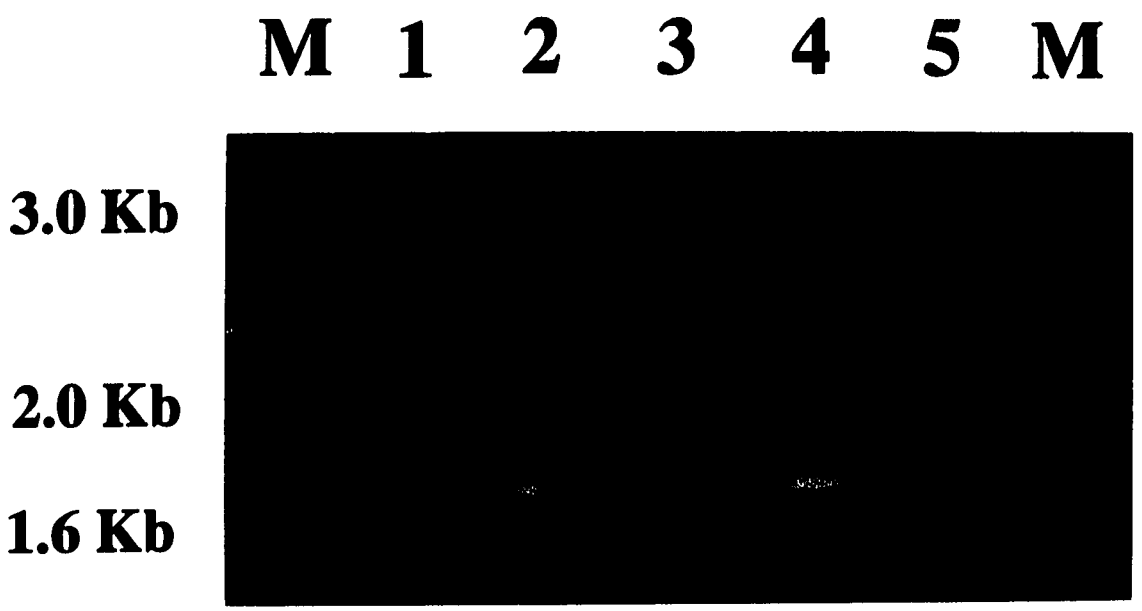
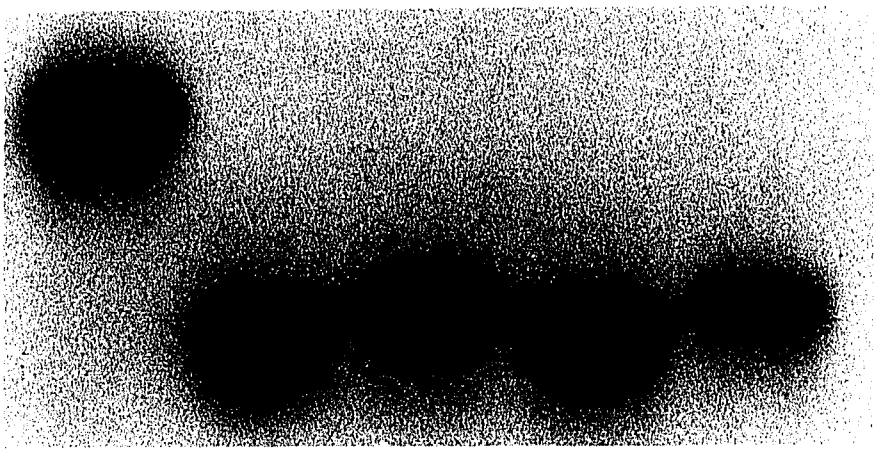

M 1 2 3 4 5 M
3.0 Kb
2.0 Kb
1.6 Kb

Figure 4. Southern blot of the PCR product from the 5'-half of the S gene of TGEV and PRCV isolates with the cDNA probes FP2 and FP1. a) The cDNA probe FP2 was labeled with [α ^{32}P]dCTP and was hybridized with the PCR products from CHV TGEV (lane 1), ISU-1 (lane 2), AR310 (lane 3), IA1894 (lane 4), LEPP (lane 5). Note that the cDNA probe FP2 hybridized with the PCR products from both TGEV and PRCV. b) The cDNA probe FP1 was labeled with [α ^{32}P]dCTP and was hybridized with the PCR products from CHV TGEV (lane 1), ISU-1 (lane 2), AR310 (lane 3), IA1894 (lane 4), LEPP (lane 5). Note that the cDNA probe FP1 hybridized only with the PCR products from TGEV, the PRCV PCR products were not detected with the cDNA probe FP1.

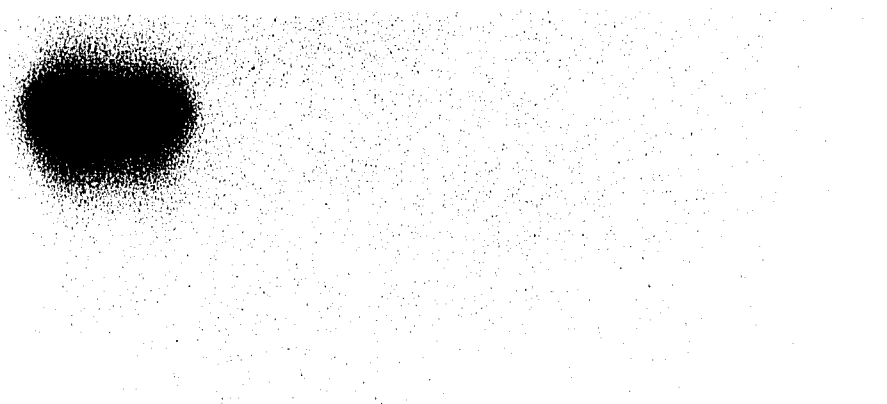
A

1 2 3 4 5



B

1 2 3 4 5



SEQUENCE COMPARISON OF THE SPIKE, 3, AND 3-1 GENES OF PORCINE RESPIRATORY CORONAVIRUS ISOLATES WITH VARYING PATHOGENICITY

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Abstract

Four porcine respiratory coronavirus (PRCV) isolates with varying pathogenicity were characterized. Subgenomic mRNA patterns and the nucleotide sequence of the 5' end of the S genes, the 3/3a genes, and the 3-1/3b genes of these PRCV isolates were determined and compared to other PRCV and transmissible gastroenteritis virus (TGEV) isolates. Two of the PRCV isolates, AR310 and LEPP, were shown to be more pathogenic than another PRCV isolate IA1894 in a five-week-old specific pathogen free model. Northern blot analysis of the subgenomic mRNAs produced in infected-ST cells revealed that the mRNA 2 (S) of the PRCV isolates migrated faster than the homologous species of mRNA from TGEV. Also, the number of subgenomic mRNAs produced in infected ST cells was found to vary. The PRCV isolates AR310 and LEPP produced eight subgenomic mRNA species, the same number as that produced by the Miller strain of TGEV. However, the PRCV isolates IA1894 and ISU-1 produced only seven subgenomic mRNA species. All four of the PRCV isolates were found to have a large inframe deletion in the 5' end of the S gene. The PRCV isolates AR310 and LEPP both had identical S gene deletions of 621-nucleotides starting 47 nucleotides after the S gene start site. The PRCV isolate IA1894 had a 678-nucleotide deletion 44 nucleotides after the S gene start site, whereas the PRCV isolate ISU-1 had a 681-nucleotide deletion 62

nucleotides after the S gene start site. Analysis of the 3/3a gene nucleotide sequence from the four PRCV isolates showed a high degree of variability. The 3 gene of the PRCV isolates AR310 and LEPP was preceded by a CTAAAC leader RNA binding site and the 3 gene was predicted to yield a protein of 72 amino acids, the same size as that of the virulent Miller strain of TGEV. The 3a gene of the PRCV isolate IA1894 was preceded by a CTAAAC leader RNA binding site and the 3a gene was predicted to yield a truncated protein of 53 amino acids due to a 23-nucleotide deletion in the 3a gene. The CTAAAC leader RNA binding site and ATG start codon of the 3 gene of the PRCV isolate ISU-1 was removed due to a 168-nucleotide deletion. Analysis of the 3-1/3b gene nucleotide sequence from the four PRCV nucleotides isolates also showed a rather high degree of variability. The 3-1 gene of the PRCV isolates AR310 and LEPP was preceded by a CTAAAC leader RNA binding site but was found to have a one-nucleotide deletion that was predicted to yield a truncated (63 amino acids versus 244 amino acids) 3-1 protein. The last two nucleotides of the CTAAAC leader RNA binding site preceding the 3-1 gene of the PRCV isolate IA1894 were removed by a five-nucleotide deletion but the 3-1 gene was predicted to encode for a protein of 244 amino acids. The 3-1 gene of the PRCV isolate ISU-1 was preceded by a CTAAAC leader RNA binding site but was found to have a 117-nucleotide inframe deletion that was predicted to yield a truncated (205 amino acids versus 244 amino acids) 3-1 protein. The PRCV isolates AR310 and LEPP have been shown to be more pathogenic than the PRCV isolate IA1894, which suggests that the presence of an intact 3 gene may be an important virulence determinant in PRCV.

Introduction

Porcine respiratory coronavirus (PRCV) and transmissible gastroenteritis virus (TGEV) are members of the *Coronaviridae* family of viruses (18,21). Coronaviruses are pleomorphic enveloped viruses with a positive-sense single stranded RNA genome (21). Transmissible gastroenteritis virus causes severe diarrhea with a high mortality in neonatal swine (18). Transmissible gastroenteritis virus replicates in and destroys the villus enterocytes of the small intestinal epithelium which causes the subsequent malabsorption and dehydration characteristic of transmissible gastroenteritis (TGE) (18). Transmissible gastroenteritis virus has also been shown to replicate in respiratory tissue of infected swine. Porcine respiratory coronavirus is believed to be a mutant of TGEV as it has been shown to be antigenically and genetically related to TGEV but has a selective tropism for respiratory tissue with very little to no replication in intestinal tissue of infected swine (10, 15). Porcine respiratory coronavirus is widespread in Europe where it was first detected (15). Porcine respiratory coronavirus has also been found in the United States, but the extent of its prevalence is not known (7,14,26). Because TGEV and PRCV are closely related and yet display differences in tissue tropism and pathogenicity, they can serve as useful models for the study of coronavirus genes involved in tropism and virulence.

The replication scheme of coronaviruses is characterized by the development of a 3'-terminal nested set of subgenomic mRNAs in infected cells (21). The subgenomic mRNAs, with the exception of the smallest, are polycistronic in nature yet only unique 5' ORF is translated (21). Seven or eight subgenomic mRNAs are synthesized during TGEV replication in infected cells (21,25). The full-length genomic mRNA 1 encodes for a RNA-dependent RNA polymerase (21). The spike (S) protein is encoded by subgenomic mRNA 2 (21). Two nonstructural proteins, 3 and 3-1, are encoded by the

subgenomic mRNAs 3 and 3-1 respectively, for the Miller strain of TGEV (21,24,27). The homologous genes found in the Purdue and FS772/70 strains of TGEV are encoded by a single mRNA species and are designated genes 3a and 3b (2,9). The small integral membrane (sM) protein is associated with the viral envelope and is encoded by subgenomic mRNA 4 (5). The integral membrane protein (M) and nucleocapsid (N) proteins are encoded by subgenomic mRNAs 5 and 6, respectively (21). The subgenomic mRNA 7, the smallest subgenomic mRNA, is the only monocistronic subgenomic mRNA and has been shown to encode for a possible DNA binding protein in infected cells (2,4). Preceding each large potential ORF is the conserved hexameric sequence of CTAAAC, which is thought to serve as a consensus leader RNA binding site in the transcription of the subgenomic mRNAs from a full-length negative sense template (21).

Previous studies of the genetic structure of PRCV have shown that all of the isolates have two unique characteristics. First, the S gene of PRCV contains a large inframe deletion ranging from 672- to 681-nucleotides in length (1,8,10,17,24). This deletion in the S gene results in a smaller S glycoprotein. Second, all of the PRCVs analyzed thus far have had the CTAAAC consensus leader RNA binding site preceding the 3 gene altered or partially deleted (10,12,17,24). Thus the subgenomic mRNA 3 is not detected in PRCV infected cells (10). Also, the 3 gene of the European PRCVs contains several small deletions that render the 3 gene to a psuedogene (12,17). One PRCV isolate from the United States, Ind/89, was found to have an intact 3 gene but the CTAAAC consensus leader RNA binding site had been altered to CTAAAT, thus making the subgenomic mRNA 3 of Ind/89 undetectable in infected cells (24). The presence of a large deletion in the 5' end of the S gene is thought to play a role in determining the tissue tropism of PRCV (13,20). In the case of TGEV, the genes 3 and 3-1 (formerly

designated as ORF A and ORF B respectively) have been hypothesized to be important in the virulence and pathogenesis of TGEV infection (25). It is interesting to note that all previously described PRCV isolates have been demonstrated to have an altered or deleted 3 gene and that these PRCVs all cause minimal to no apparent clinical disease in swine.

In this study, my colleagues and I have analyzed the viral mRNAs from four PRCV isolates by Northern blot analysis and nucleotide sequencing of the 5' end of the S gene and the 3 and 3-1 genes. The size and location of the S gene deletion of the PRCV isolates was also determined as my colleagues and I had previously shown that two of the PRCV isolates in this study, AR310 and LEPP, had smaller deletions in the 5' end of the S gene when compared to another United States PRCV isolate designated ISU-1. Also, Halbur et al. (1994) have recently shown that three of the PRCV isolates analyzed in this study vary in pathogenicity. The PRCV isolates AR310 and LEPP caused moderate bronchointerstitial pneumonia in five-week-old specific pathogen free (SPF) pigs whereas the PRCV isolate IA1894 caused only a very mild bronchointerstitial pneumonia in SPF pigs. Since the 3 and 3-1 genes have been hypothesized to determine the virulence of TGEV, my colleagues and I examined the 3 and 3-1 genes of the two pathogenic and the one nonpathogenic PRCV isolates to determine if the 3 and 3-1 genes of these PRCV isolates could be related to the differences in virulence exhibited by the PRCV isolates.

Materials and Methods

Virus isolation

The PRCV isolate AR310 was isolated from the small intestines of a pig from a swine herd in Arkansas with endemic TGE as previously described (22). Briefly, approximately 10 percent suspensions of the intestinal sample was prepared in 0.05 M

phosphate buffered saline (pH 7.4) and clarified by centrifugation at 1000 x g for 10 minutes. The supernatants were harvested, passed through a 0.45 μm filter (Costar, Cambridge, MA) and stored at -70°C until viral isolation was attempted. The swine testicular (ST) cell line was used to isolate and propagate the viruses in this study and were maintained as previously described (23). Four-day-old ST cells in 25 cm^2 flasks (Costar, Cambridge, MA) were treated with minimum essential medium (MEM) containing DEAE-dextran (50 μg/ml) (Sigma, St. Louis, MO) at 37°C for 30 minutes before the intestinal tissue filtrate was added. Prior to inoculation onto ST cell monolayers, 0.2 ml of the intestinal filtrate was mixed with 0.8 ml of a 1:10 dilution of porcine anti-enterovirus (Group 8C) hyperimmune serum (virus neutralization titer of 1:1600) in MEM with 2 percent fetal bovine serum (FBS) and antibiotics (penicillin 20,000 U/ml, streptomycin 20,000 μg/ml, and amphotericin B 50 μg/ml) (GIBCO BRL, Grand Island, NY), and were incubated at 37°C for one hour. The ST cell monolayers were inoculated with the entire 1 ml of virus and anti-enterovirus hyperimmune serum mixture and the inoculum was adsorbed onto the ST cells for one hour at 37°C, after which additional MEM with 2 percent FBS plus antibiotics was added. The cultures were incubated at 37°C for 48 hours and observed daily for cytopathic effect (CPE). After 48 hours, whether or not CPE was evident, all cultures were frozen and thawed three times, clarified by centrifugation at 1000 x g for 10 minutes, and used as inoculum for the next passage. CPE was evident at the third passage in ST cells for AR310.

The other two PRCV isolates in this study, IA1894 and LEPP, were isolated from nasal swabs from swine from herds that had antibodies to TGEV but had not presented evidence of diarrhea, and thus were suspected of having a PRCV infection (22). Nasal swabs were collected and placed into one ml of MEM with 2 percent FBS and antibiotics and mixed for 20 seconds. Two hundred μl of the nasal swab MEM was

inoculated onto ST cells. CPE was present on the first passage in ST cells for both IA1894 and LEPP. All three isolates were plaque purified a total of three times and stock virus was stored at -70°C.

The gut-passaged virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, IA), also referred to as CHV TGEV, was used as a standard TGEV strain in this study. The PRCV isolate ISU-1 was received as a plaque-purified preparation and was kindly provided by Dr. Howard Hill (Iowa State University Veterinary Diagnostic Laboratory)(7).

RNA isolation

ST cells were infected at a multiplicity of infection of approximately 0.1 PFU/cell with CHV TGEV, and the PRCV isolates AR310, ISU-1, IA1894, or LEPP. At 19 hours post infection, the medium was removed and the total RNA was isolated from the infected ST cell monolayers by a rapid guanidinium thiocyanate method (Stratagene, La Jolla, CA). The RNA was washed with 70% ethanol and dissolved in diethylpyrocarbonate (DEPC) -treated distilled water and stored at -70°C.

cDNA synthesis and PCR amplification

First strand cDNA was made from total RNA from infected ST cells by avian myeloblastosis virus reverse transcriptase by using random oligonucleotide primers (Invitrogen, San Diego, CA). The cDNA-RNA hybrids were amplified by PCR using Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) using the following primers (Table 1) and cycles.

The S genes of TGEV and the PRCV isolates were amplified by using the primers 21209 and 060704 (Figure 2) under the following parameters: 1 cycle of 1

minute at 94°C, 1 minute at 48°C, and 5 minutes at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C; followed by 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 5 minutes at 72°C in a DNA thermal cycler (Coy Corporation, Grass Lake, MI).

The 3 and 3-1 gene regions of TGEV and PRCV were amplified by using the primers 538 and 622 (Figure 2) under the following parameters: 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 48°C, and 2 minutes at 72°C; followed by 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C in a DNA thermal cycler.

A 462 bp PCR product from the 3' end of the TGEV genome was amplified by using the primers 068 and 069 under the following parameters: 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 48°C, and 1 minutes at 72°C; followed by 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C in a DNA thermal cycler.

The 5' end of mRNA 3 was amplified by using the primers 119 and 118, and the 5' end of mRNA 3-1 was amplified by using the primers 119 and 048. Both reactions were amplified under the following parameters: 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 48°C, and 1 minutes at 72°C; followed by 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C in a DNA thermal cycler.

Cloning and sequencing

The PCR products of the 5'-half of the S gene and 3 and 3-1 genes from TGEV and PRCV were separated on a 2% NuSieve GTG (FMC Bioproducts, Rockland, ME) agarose gel and then purified from the agarose gel by the Magic PCR prep method

(Promega, Madison, WI). The purified PCR products were then digested with *Eco*RI and *Bam*HI and cloned in the phagemid vector pKS+. Five clones of the 5'-half of the S gene and the 3 and 3-1 gene regions from each virus were sequenced. The 3 and 3-1 gene PCR products were also sequenced directly with specific oligonucleotide primers.

Northern blot hybridization analysis

Forty μg of total RNA was denatured with formaldehyde and formamide (19) and separated by electrophoresis in a 1.5% SeaKem (FMC Bioproducts, Rockland, ME) agarose gel . After electrophoresis, the agarose gel was treated with 0.05 *N* NaOH and 1.5 *M* NaCl, neutralized with 0.1 *M* Tris-HCl (pH 7.5) and 1.5 *M* NaCl, washed twice in 10x SSC (1x SSC is 0.15 *M* NaCl plus 15 m*M* sodium citrate), and then the RNA was transferred by using a Posiblot pressure blotter (Stratagene, La Jolla, CA) with 10x SSC to nylon membranes (Magna NT, Micron Separations Inc., Westboro, MA). After the transfer was complete, the membranes were baked at 80°C for 2 hours to fix the RNA. The membranes were prehybridized for 2 hours in a solution containing 50% formamide, 5x SSPE (1x SSPE is 0.18 *M* NaCl, 10 m*M* sodium phosphate, and 1 m*M* EDTA) 4x Denhardt's solution, 0.3% sodium dodecyl sulfate (SDS), and sonicated salmon sperm DNA (30 μg/ml) at 42°C. The probe used in the hybridization procedure was a 462 bp PCR product amplified with the primers 068 and 069 (Table 1). This PCR product was amplified from TGEV cDNA, gave a probe specific for the 3' end of TGEV, and was thus suitable for use as a probe in a Northern blot to detect TGEV and PRCV subgenomic mRNAs. One hundred twenty-five ng of the 3' end PCR product was labeled with [α ^{32}P]dCTP (ICN Biochemicals, Costa Mesa, CA) in the presence of random hexamer primers and Klenow fragment of DNA polymerase I (Amersham Corporation, Arlington Heights, IL). Unincorporated [α ^{32}P]dCTP was removed by

passing the labeled DNA through a Sephadex G-50 column (Boehringer Mannheim, Indianapolis, IN). The labeled 3' end probe was then heated to 100°C, cooled on ice, and then added to the prehybridization reaction and allowed to hybridize overnight. The membranes were washed once in 2xSSC-0.3% SDS at room temperature, twice in 2xSSC-0.3% SDS at 65°C, and once with 0.2x SSC at room temperature. Autoradiographs were made by exposing X-ray film (RX film, Fuji Photo Film Co., Japan) to the membranes.

Analysis of the 5' end of mRNA 3 and mRNA 3-1

PCR products from the 5' end of mRNA and mRNA 3-1 were generated using a method similar to that used by Page et al. (1991). Utilizing a primer (119) complementary to the TGEV and PRCV leader RNA sequence as determined by Page et al. (1990), and primers from either the 5' end of the 3/3a gene (118) or the 5' end of the 3-1/3b gene(048), PCR products would be generated if the leader sequence had bound to the CTAAAC consensus leader binding site preceding the respective gene (Figure 10). Using the primers 119 and 118, a PCR product would be generated if the leader RNA was joined upstream of the 3/3a gene. Alternatively, using the primers 119 and 048, two PCR products would be generated if the leader RNA was joined upstream of both the 3/3a and 3-1/3b genes, whereas only one PCR product would be generated if the leader RNA was joined upstream of either 3 gene or 3-1 gene (Figure 11). Amplified PCR products were then separated by electrophoresis in a 1.5% SeaKem (FMC Bioproducts, Rockland, ME) agarose gel and stained with ethidium bromide.

Results

Analysis of subgenomic mRNAs

Coronaviral subgenomic mRNAs in ST cells infected with CHV TGEV or the PRCV strains were examined by Northern blot analysis using a probe specific for the 3' end of TGEV (Figure 1). The virulent Miller CHV TGEV had 8 mRNA species present as has been previously reported (27). The PRCV isolates AR310 and LEPP also had 8 mRNA species present, whereas the PRCV isolates IA1894 and ISU-1 only had 7 mRNA species. The migration pattern of the mRNA species 4 through 7 of all four PRCV isolates were similar to that of the virulent TGEV. The mRNA 2 species of the PRCV isolates examined migrated faster than the TGEV equivalent, indicating that a large deletion was present. The PRCV isolates AR310 and LEPP and the virulent CHV TGEV all had the mRNA species 3 and 3-1 present. However, the PRCV isolates IA1894 and ISU-1 had only one mRNA species present in the area in which the mRNAs 3 and 3-1 were detected for AR310, LEPP, and CHV TGEV. The PRCV isolate IA1894 had a mRNA species of approximately 3.61 kb that migrated at a slightly faster rate than the mRNA 3 of AR310, LEPP, and CHV TGEV. The PRCV isolate ISU-1 had a mRNA species of approximately 3.35 kb that migrated at a slightly faster rate than the mRNA 3-1 of AR310, LEPP, and CHV TGEV. The mRNA 1 appeared to migrate at the same rate for all of the isolates.

Analysis of the 5'-half of the S gene PCR products

To determine the size and location of the deletion present in the 5' end of the S gene of the PRCV isolates, the genome region from the 3' end of the polymerase gene (primer 021209) to the middle of the S gene (primer 060704) was amplified by PCR (Figure 2). Following amplification, a PCR product of the expected 2.4 kb size was

present for CHV TGEV. However, all the PRCV isolates had PCR products that migrated faster than the PCR product of TGEV indicating that deletions in the range of approximately 600- to 700-nucleotides were present. The deletions in the 5' end of the S genes varied in size as previously reported (22). The PCR products of the 5'-half of the S gene from the PRCV isolates were cloned and sequenced to map the size and location of the deletions present in the 5' end of the S gene. Alignment of the nucleotide sequence from the different isolates is shown in Figure 3. The PRCV isolates AR310 and LEPP had identical deletions of 621-nucleotides beginning 47 nucleotides after the ATG start of the S gene. The PRCV isolate IA1894 had a 678-nucleotide deletion 44 nucleotides after the S gene ATG. The 681-nucleotide deletion in the 5' end of the S gene of PRCV ISU-1 was found to occur 62 nucleotides after the S gene ATG start. The S gene deletion found in PRCV ISU-1 is the same size and in the same location as had been previously determined by other researchers (8) and of that of another U.S. PRCV isolate Ind/89 (24). A schematic diagram showing the various S gene deletions of the PRCV isolates is shown in Figure 4.

Figure 5 shows the alignment of the predicted amino acid residues of the S gene from the PRCV isolates. The predicted S protein signal peptide cleavage site of IA1894 differs from that of TGEV or the other PRCV isolates. However, the signal peptide region of the PRCV isolate IA1894 still retains a core of hydrophobic amino acid residues that are essential for recognition by signal peptidase.

Analysis of the 3/3a gene and 3-1/3b gene regions

The PCR products (primers 538 and 622) of the 3 and 3-1 gene regions of the PRCV isolates AR310 and LEPP, and of CHV TGEV were the expected size of 1.2 kb (Figure 6). The PCR product from the PRCV isolate IA1894 migrated slightly faster

indicating that a small deletion of approximately 20-nucleotides was present. However, the PCR product from the PRCV isolate ISU-1 migrated much faster, indicating that approximately 290-nucleotides were deleted. The alignment of the nucleotide sequence from the 3/3a and 3-1/3b gene regions of CHV TGEV, and the PRCV isolates AR310, LEPP, IA1894, and ISU-1 is shown in Figure 7. The nucleotide sequences of the TGEV isolates PP3 (Miller) (27), FS772/70 (2), and Purdue (9) and of the PRCV isolates 86/137004 (12) and RM4 (17) are included in Figure 7 for comparison. Also, the predicted amino acid residues for the 3/3a and 3-1/3b gene regions are shown in Figures 8 and 9 respectively. Cloning and sequencing of the PCR products from the 3 and 3-1 gene region revealed that the PRCV isolates AR310, LEPP, IA1894, and ISU-1 all had a three-nucleotide deletion beginning at position 42 which was identical to that detected in the British PRCV isolate 86/137004. The 3 gene from the isolates PRCV AR310, PRCV LEPP, and CHV TGEV was preceded by a consensus CTAAAC leader RNA-binding site. The 3-1 gene for PRCV AR310, PRCV LEPP, and CHV TGEV also was preceded by a consensus CTAAAC leader RNA-binding site. Analysis of the 3-1 gene of PRCV AR310 and PRCV LEPP revealed that a one-nucleotide deletion was present at position 580. This one-nucleotide deletion causes a frameshift resulting in early termination of the 3-1 protein. The predicted 3-1 protein for PRCV AR310 and LEPP is 64 amino acids in length, 180 amino acid residues shorter than the predicted 3-1 protein for CHV TGEV and the other PRCV isolates. (Figure 9). Analysis of PRCV IA1894 revealed that there was a consensus CTAAAC leader RNA-binding site preceding the 3a gene. Of note, the PRCV IA1894 3a gene contained a 23-nucleotide deletion beginning at position 287 that would yield an early stop codon that would give rise to a truncated 3a protein (Figure 7). The truncated 3a protein of PRCV IA1894 would consist of 53 amino acids as compared to the 72 amino acids present for PRCV

AR310, PRCV LEPP, and CHV TGEV 3 protein (Figure 8). The consensus CTAAAC leader RNA-binding site preceding the 3b gene of PRCV IA1894 was altered due to a five-nucleotide deletion that removed the last two nucleotides of the consensus sequence, thus making RNA 3-1 undetectable in PRCV IA1894 infected cells by Northern blot. However, the 3-1 gene of PRCV IA1894 had a ATG start codon present and the encoded 3b protein was predicted to be intact. Thus the PRCV isolate IA1894 synthesizes a mRNA 3 similar to that of the Purdue and FS772/70 strains of TGEV in that the mRNA 3 is thought to code for both 3a and 3b proteins. The PRCV isolate ISU-1 was found to have a 168-nucleotide deletion present at position 53 that removed both the consensus CTAAAC leader RNA-binding site and the ATG start codon of the 3 gene. Further downstream at position 118, a five-nucleotide deletion in the 3a gene of PRCV ISU-1 was also detected. The CTAAAC leader RNA-binding site preceding the 3-1 gene of PRCV ISU-1 was present, however, the 3-1 gene had a 117-nucleotide inframe deletion beginning at position 329 that would remove 39 amino acids from the 244 amino acids found in the intact 3-1 protein of TGEV.

Analysis of the 5' end of mRNA 3 and mRNA 3-1

PCR products from the 5' end of mRNA 3 were generated for CHV TGEV and the PRCV isolates AR310, LEPP, and IA1894 which confirms the joining of the leader RNA to the CTAAAC leader RNA binding site preceding the 3/3a gene (Figure 12). The PCR products from CHV TGEV, AR310 and LEPP were the expected size of 310 bp. The PCR product from IA1894 migrated slightly faster with a PCR product of 287 bp due to a 23-nucleotide deletion in the coding region of the 3a gene. As expected, no PCR product was evident from the PRCV isolate ISU-1 due to the CTAAAC leader RNA binding site preceding the 3 gene being removed by a 168-nucleotide deletion.

Two PCR products of 660 and 370 bp were amplified from CHV TGEV, AR310 and LEPP indicating that the leader sequence had bound to the CTAAAC leader RNA binding site preceding the 3 gene and 3-1 gene (Figure 13). The PRCV isolate IA1894 had only a single PCR product of 632 bp evident, which is consistent with the leader RNA joining the CTAAAC leader RNA binding site preceding the 3a gene and a 23-nucleotide deletion in the 3a gene. The leader RNA did not bind to the CTAAAC leader RNA binding site preceding the 3b gene due to a five-nucleotide deletion removing the last two nucleotides of the CTAAAC sequence. The PRCV isolate ISU-1 had only a single PCR product of 253 bp evident, which is consistent with there being a CTAAAC leader RNA binding site preceding the 3-1 gene and a 117-nucleotide inframe deletion present in the coding region of the 3-1 gene, and the loss of the CTAAAC leader RNA binding site preceding the 3 gene due to the 168-nucleotide deletion present in the 3 gene region.

Discussion

Studies in this report show that three new PRCV isolates AR310, LEPP, and IA1894 are unique from previously reported PRCV isolates. The size and locations of the S gene deletions found in these PRCV isolates were shown to vary. Two of the PRCV isolates, AR310 and LEPP, have the smallest S gene deletions (621-nucleotides) found thus far among PRCV isolates. The size of the S gene deletions for other PRCV isolates from Europe is 672-nucleotides (1, 17), and the size of the S gene deletion of other PRCV isolates from the United States is 681-nucleotides (8, 24). The PRCV isolates AR310 and LEPP had identical deletions of 621-nucleotides beginning 47 nucleotides after the ATG start of the S gene. The PRCV isolate IA1894 had a 678-nucleotide deletion 44 nucleotides after the S gene ATG. The 681-nucleotide deletion in

the 5' end of the S gene of PRCV ISU-1 was found to occur 62 nucleotides after the S gene ATG start. The S gene deletion found in PRCV ISU-1 is the same size and in the same location as had been previously determined by other researchers (8) and as that of another U.S. PRCV isolate Ind/89 (24). Even though the deletions in the 5' end of the S gene of PRCV AR310 and PRCV LEPP are smaller than that of other PRCV isolates, the area deleted encompasses the S protein amino acid residues of 92, 94, 218, and 219 that were predicted by Sanchez et al. (1992) to be important in predicting tissue tropism of PRCV and TGEV isolates.

Also of significance, the PRCV isolates AR310 and LEPP are the first to have been found to have an intact and functional 3 gene. The sequence information from all other PRCV isolates previously sequenced have shown that the 3 gene has deletions or alterations that render the 3 gene nonfunctional. As the sequence information from the PRCV isolate IA1894 indicates, this particular isolate is more in keeping with the previously described PRCV isolates in that there is a 23-nucleotide deletion in the 3a gene that would result in a truncated 3a protein. The PRCV isolate ISU-1 also fits into the category of previously described PRCV isolates since PRCV ISU-1 had its 3 gene altered to a psuedogene due to a 168-nucleotide deletion in the 3 gene that removed both the CTAAAC leader RNA-binding site and the ATG start site of the 3 gene.

Surprisingly, the 3-1 gene coding region of the PRCV isolates AR310 and LEPP contained a one-nucleotide deletion and is predicted to cause a frameshift in the 3-1 gene resulting in a truncated 3-1 protein of 64 amino acids. Whether this truncated 3-1 protein would still be functional is not known, but it may be possible that this truncated 3-1 protein may still have the necessary domains to be functional. However, the 3-1 gene of all other PRCV isolates shows that the 3-1 gene is preceded by a CTAAAC leader RNA binding site and the following ORF was intact. However, the 3b gene of the PRCV

isolate IA1894 was preceded by an altered CTAAAC leader RNA-binding site but the 3b protein was found to be intact and nearly identical to that of TGEV. This would mean, with the exception of the 23-nucleotide deletion present in 3a gene, that the PRCV IA1894 mRNA 3 is like that of Purdue and FS772/70 strains of TGEV in that the mRNA 3 is thought to code for both the 3a and 3b proteins (2,9). Additionally, the 3-1 gene of PRCV ISU-1 was found to have a 117-nucleotide inframe deletion at amino acid residue 32, that would effectively remove 39 out of a possible 244 amino acid residues found in the intact 3-1 protein. Whether this 117-nucleotide inframe deletion in the 3-1 gene of PRCV ISU-1 would still yield a functional protein is not known.

Since both the 3 and 3-1 genes of the PRCV isolate ISU-1 are altered, it exhibits the same genetic characteristics as the SP mutant of TGEV which is lacking the entire 3 gene and part of the 3-1 gene (previously described as regions A and B, respectively) due to a 462-nucleotide deletion (25). By using S1 nuclease analysis, Wesley et al. (1990) demonstrated that the SP TGEV S gene was similar to that of virulent TGEV and contained no large deletions in the SP S gene characteristic of PRCV. The SP TGEV was not able to replicate in the villus epithelium of the gut of neonatal pigs and thus did not cause enteric disease. However, using IFA the SP TGEV was shown to replicate in the cells of the lamina propria of neonatal pigs infected with SP TGEV. It is not known if the SP TGEV was able to replicate in the lungs and other respiratory tissue of SP TGEV infected pigs. The SP TGEV was able to replicate in ST cells, but exhibited a SP morphology. The lack of intact 3 and 3-1 genes in PRCV ISU-1 does not affect its ability to replicate in ST cells or its plaque morphology, as PRCV ISU-1 can reach titers exceeding 10^6 pfu/ml and the plaque size is approximately 5 mm (data not shown). The lack of an intact 3a gene does not affect the ability of PRCV IA1894 to replicate in ST cells as it can reach titers exceeding 10^6 pfu/ml and the plaque size is approximately 5

mm (data not shown). In a similar manner, the same can be said for the PRCV isolates AR310 and LEPP as these isolates readily grow to titers exceeding 10^6 pfu/ml and the plaque size is approximately 5 mm (data not shown). Wesley et al. (1990) proposed that one of the functions of the 3 and 3-1 genes was determination of plaque size in cell culture. Since the PRCV isolate ISU-1 is missing the 3 gene just like all other PRCV isolates (except AR310 and LEPP), but has a truncated 3-1 protein and can still form large plaques in cell culture, the ability of the PRCV isolate ISU-1 to form large plaques may indicate that its smaller 3-1 protein is still functional. The 3-1 gene may be responsible for determining plaque size in cell culture as the SP TGEV mutant was lacking this region, and was not able to produce large plaques in cell culture.

Studies in our laboratory (Halbur et al., 1994) have shown that the PRCV isolates AR310 and LEPP are more virulent in five-week-old SPF pig model than the PRCV isolate IA1894. Wesley et al. (1990) have hypothesized that the role of the 3 and 3-1 genes of TGEV are involved in pathogenesis and virulence. This hypothesis can be extended to PRCV. The fact that the PRCV isolates AR310 and LEPP both have an intact 3 gene, and the fact that these two isolates have been shown to cause more extensive lung lesion development and are more pathogenic than the typical PRCV isolate IA1894, supports the hypothesis that the 3 gene is important in the virulence of both TGEV and PRCV. A schematic diagram of the comparison of gene deletions, tissue tropism, and pathogenicity of TGEV and PRCV isolates is shown in Figure 14.

The PRCV isolate IA1894, except for a 23-nucleotide deletion in the coding region of the 3a gene, has a mRNA 3 that was revealed to be very similar to that of the virulent TGEV isolates FS772/70 and Purdue. These two TGEV isolates have genes 3a and 3b present on single mRNA species, and the 3b protein is assumed to be expressed from the functionally bicistronic mRNA 3. Since the PRCV isolate IA1894 has been

determined to be less pathogenic than the PRCV isolates AR310 and LEPP, and yet the mRNA 3 of PRCV IA1894 is very similar (with the exception of the 23-nucleotide deletion) to that of two virulent TGEV isolates, this suggests that the 3 gene is important in determining the virulence of PRCV and TGEV isolates. Specifically, the region of the 3/3a protein that is deleted from IA1894 and yet is still conserved in the PRCV isolates AR310 and LEPP and the virulent TGEV isolates CHV, PP3, FS772/70, and Purdue is a region of four amino acid residues in length designated KLGL. These four amino acid residues may comprise an important motif or domain in the 3/3a protein that is essential for virulence in the animal host. The function of the 3/3a protein and its role in virulence needs to be clarified with further study.

Future studies to clarify the role of the 3/3a gene in virulence of PRCV and TGEV isolates would best be addressed by the correction of 3/3a gene defects by RNA recombination. Also, the use of RNA recombination to introduce a TGEV-like S gene to repair the deleted S gene regions of PRCV isolates would also be useful to further clarify the role of the S gene and its smaller S protein product in the change of tissue tropism of PRCV from the enteropathogenic TGEV. The PRCV isolates, AR310, LEPP, and IA1894, would be excellent candidates for future experiments to correct its 3a and S gene deletions. For example, if the S gene deletion of AR310 or LEPP was corrected to a TGEV-like S gene, would the S gene-corrected AR310 or LEPP become enteropathogenic? Additionally, if the PRCV isolate IA1894 had the 23-nucleotide deletion present in the 3a gene corrected, would the 3a gene-corrected IA1894 cause as severe lung lesions as AR310 or LEPP in the five-week-old SPF pig model? Furthermore, if the S gene deletion of the 3a gene-corrected IA1894 was corrected by RNA recombination would the S gene-corrected-3a gene-corrected IA1894 become an enteropathogenic TGEV isolate?

From the present study, my colleagues and I can confirm, as have others, that the 3/3a genes of both TGEV and PRCV isolates are highly variable. In addition, the 3-1/3b genes of at least PRCV isolates appears to be variable also. The extent of deletions present in the PRCV isolates AR310, LEPP, IA1894, and ISU-1 allows for some intriguing speculation as to the relationship of the S, 3/3a, and 3-1/3b genes in PRCV isolates. All previously analyzed PRCV isolates have been found to have large deletion in the 5' end of the S gene accompanied by an altered or deleted 3/3a gene, while the 3-1/3b gene has remained intact. In this study, my colleagues and I have reported on additional PRCV isolates that do have large S gene deletions. However, the 3/3a and 3-1/3b genes were more variable in that two of the PRCV isolates (AR310 and LEPP) are predicted to have intact 3 proteins of 72 amino acids combined with truncated 3-1 proteins of 64 amino acid residues. Another PRCV isolate (IA1894) is predicted to have a truncated 3a protein of 55 amino acids combined with an intact 3-1 protein of 244 amino acids. Also, the PRCV isolate (ISU-1) has the entire 3 protein is missing combined with a smaller 3-1 protein of 205 amino acid residues. It has been suggested by Laude et al. (1993) that there is a possibility of a functional link between the 3/3a gene and S gene alterations of PRCV isolates that may be complementing each other. With the information presented here, the complementation of an altered 3-1/3b gene with an altered S gene may be necessary also. This mean that in order to be a PRCV isolate, there must be a deletion in the S gene (to alter tissue tropism), accompanied by an altered 3/3a gene, an altered 3-1/3b gene, or both the 3/3a and 3-1/3b genes altered. In order to exhibit the characteristic tropism of PRCV, it may be that a large S gene deletion has to be accompanied by alterations in the 3/3a, the 3-1/3b genes, or both. A large deletion in the S gene may not be compatible without these alterations. Until the polymerase region of the coronavirus genome from virulent and avirulent coronaviruses can be studied in

depth, the area of the genome most likely to be involved in determining pathogenicity will be the 3 and 3-1 genes. No function has been assigned to these proteins as of yet.

Thus, the PRCV isolates previously described have displayed two certain characteristics typical of PRCV. First, previously described PRCV isolates displayed the characteristic of a large deletion in the 5' end of the S gene ranging from 672- to 681-nucleotides. Second, these previously described PRCV isolates all have altered or deleted CTAAAC leader RNA-binding sites that result in the 3 gene being nonfunctional. Additionally, previously studied PRCV isolates all have various size deletions ranging from five- to 36-nucleotides present in the putative 3 gene (12,17). The PRCV isolates AR310 and LEPP can thus be described as "uncharacteristic" PRCV isolates in that they have a smaller S gene deletion (621-nucleotides) and the 3 gene is predicted to be intact and functional and yet these isolates exhibit a tropism for respiratory tissue. It is believed that PRCV evolved from TGEV. The PRCV isolates AR310 and LEPP will be useful in understanding the origins of PRCV as they may represent intermediate isolates in the evolution of PRCV from TGEV.

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TABLE 1. TGEV specific oligonucleotide primers used in PCR to amplify regions of PRCV and TGEV

Primer				Nucleotide localization
Name		Sequence		
021209	5'	gggaattcgGGGTAAGTTGCTCATTAGAAA	3'	56-76 [a]
060704	5'	ggggatccGCAGTGCCACGAGTCCTATCAT	3'	2462-2483 [a]
538	5'	gggggaattcCTATTGAAAAAGTGCACGTC	3'	30-49 [b]
622	5'	gggggggatccAATGATGCTAATGACCATTC	3'	1199-1218 [b]
068	5'	CGAGATGCTCGTCTTCCTCCATGC	3'	3403-3426 [c]
069	5'	CTAGATCCAGACGTTAGCTCTTCC	3'	3841-3864 [c]
119	5'	GCTATATCTCTTCTTTTACTT	3'	29-49 [d]
118	5'	TTTGTGTGTTTACTTCTTCA	3'	314-333 [e]
048	5'	GCATAGGTCCTAAAAGTGTCATTG	3'	662-685 [e]

[a] From the sequence of Rasschaert and Laude (1987).

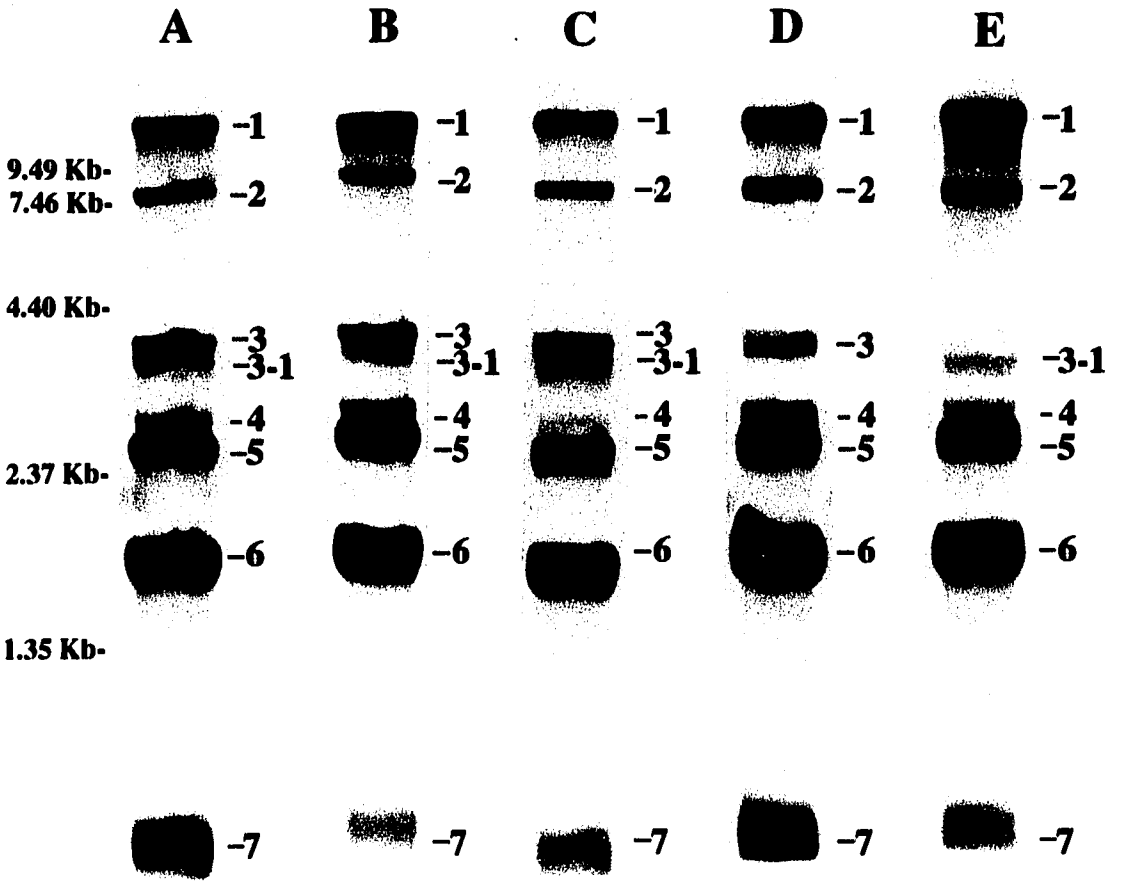
[b] From the sequence of Wesley et al., (1989).

[c] From the sequence of Rasschaert et al., (1987).

[d] From the sequence of Page et al., (1990).

[e] From CHV TGEV nucleotide sequence, this study.

Figure 1. Northern blot analysis of TGEV and PRCV subgenomic mRNAs. Total RNA from TGEV or PRCV infected ST cells was separated in a 1.5% agarose and transferred to a nylon membrane. A PCR product from the 3' end of the TGEV genome was amplified with primers 068 and 069 and was labeled with [α ^{32}P]dCTP and was used to detect TGEV and PRCV subgenomic mRNAs in a Northern blot. PRCV AR310 (lane A), CHV TGEV (lane B), PRCV LEPP (lane C), PRCV IA1894 (lane D), and PRCV ISU-1 (lane E). Note that mRNA 2 of all the PRCV isolates migrated faster than the mRNA 2 of CHV TGEV. Also, note that PRCV AR310, PRCV LEPP, and CHV TGEV all have mRNA 3 and 3-1 present. The PRCV isolate IA1894 had only one mRNA species of 3.61 kb corresponding to mRNA 3 present. The PRCV isolate ISU-1 had only one mRNA species of 3.35 kb corresponding to mRNA 3 present.


A
B
C
D
E
9.49 Kb-
7.46 Kb-
4.40 Kb-
2.37 Kb-
1.35 Kb-
-1
-2
-3
-3-1
-4
-5
-6
-7

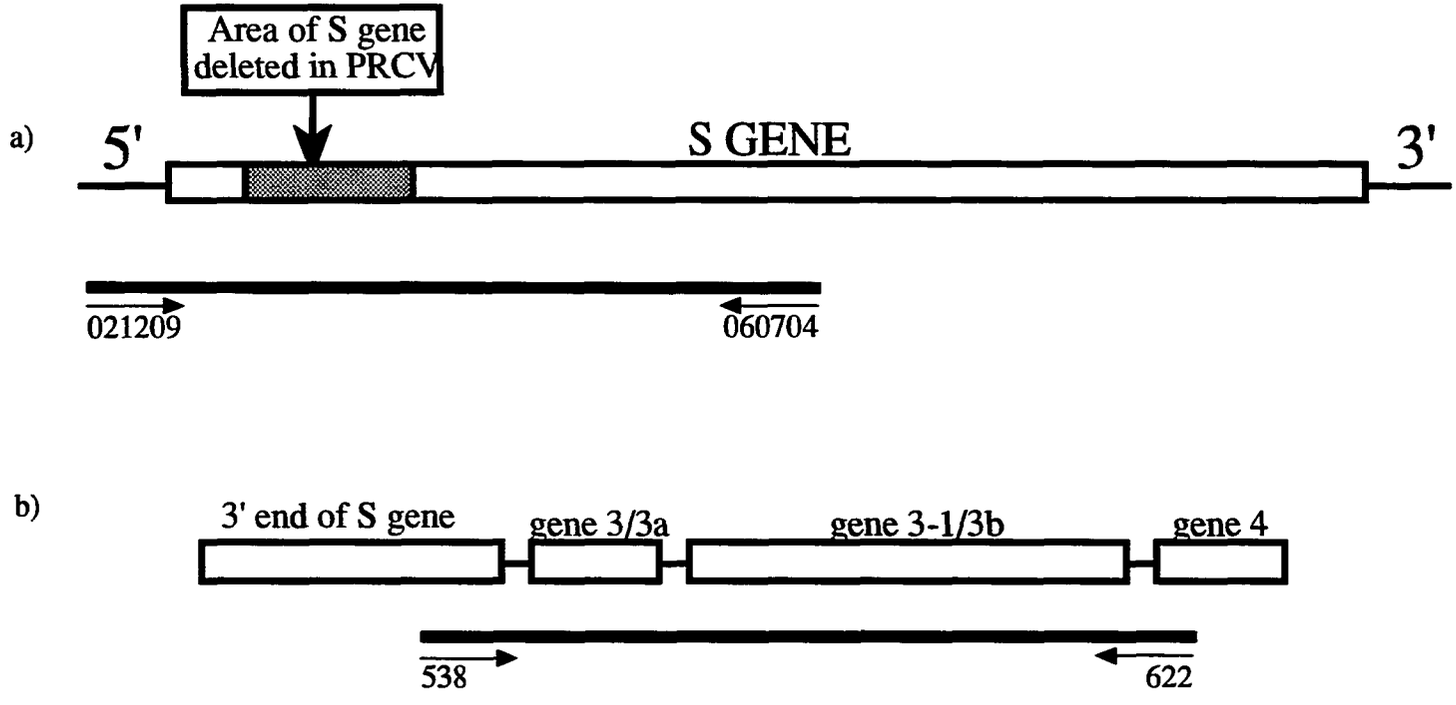


Figure 2. Schematic diagram of the regions of TGEV and PRCV isolates amplified by PCR for nucleotide sequence analysis in this study. The gray shaded area in the S gene represents the area that is deleted in PRCV isolates. a) The 5'-half of the S gene of TGEV and PRCV isolates were amplified with the primers 021209 and 060704 using conditions described in the text. b) The 3/3a and 3-1/3b gene regions of TGEV and PRCV isolates were amplified with the primers 538 and 622 using conditions described in the text.

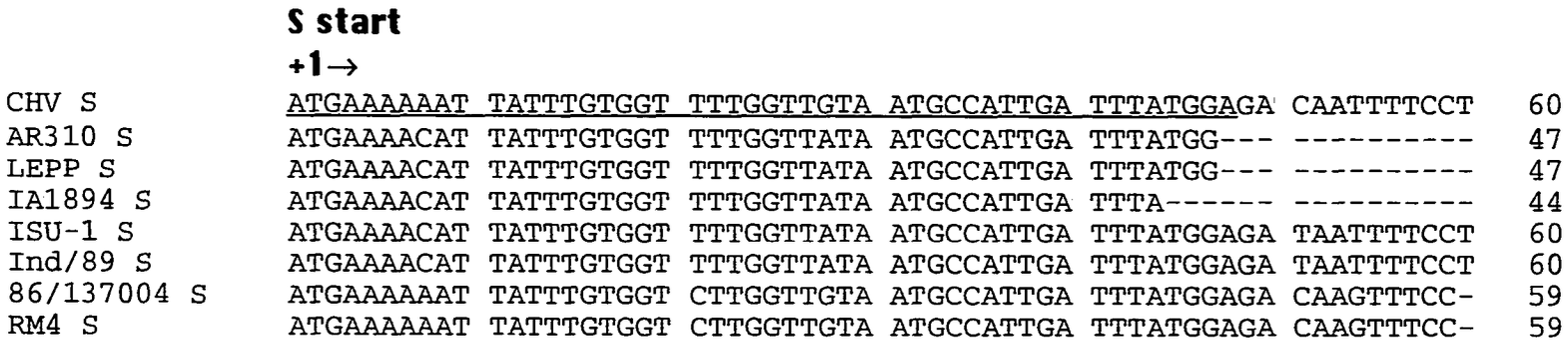


Figure 3. Alignment of the nucleotide sequence from the 5' end of the S gene of CHV TGEV and the PRCV isolates AR310, IA1894, ISU-1, Ind/89, 86/137004, and RM4. The PRCV isolates AR310 and LEPP had identical S gene deletions of 621 nucleotides present. The PRCV isolate IA1894 had a S gene deletion of 678 nucleotides present. The PRCV isolates ISU-1 and Ind/89 both had identical deletions of 681nucleotides present. The PRCV isolates 86/137004 and RM4 both had identical deletions of 672 nucleotides present. The start codon of the S gene is marked with +1→. The nucleotides comprising the predicted signal peptide region of the TGEV S gene as determined by Rasschaert and Laude (1987) are underlined. The nucleotide sequences of the TGEV isolate CHV and of the PRCV isolates AR310, LEPP, IA1894, and ISU-1 were determined in this study. The other nucleotide sequences presented in this table were previously reported (Ind/89, Wesley et al., 1991; 86/137004 PRCV, Page et al., 1991; and RM4 PRCV, Rasschaert et al., 1990).

```
CHV S          TGTTCTAAAT TGACTAATAG AACTATAGGT AACCATTGGA ATCTCATTGA AACCCTCCTT  120
AR310 S        ---------- ---------- ---------- ---------- ---------- ----------   47
LEPP S         ---------- ---------- ---------- ---------- ---------- ----------   47
IA1894 S       ---------- ---------- ---------- ---------- ---------- ----------   44
ISU-1 S        TG-------- ---------- ---------- ---------- ---------- ----------   62
Ind/89 S       TG-------- ---------- ---------- ---------- ---------- ----------   62
86/137004 S    ---------- ---------- ---------- ---------- ---------- ----------   59
RM4 S          ---------- ---------- ---------- ---------- ---------- ----------   59
```

```
CHV S          ACGCTTGTAG ACCTTTGGTG GTTTAATCCT GTTTATGATG TCAGTTATTA TAGAGTTAAT  720
AR310 S        --------AG ACCTTTGGTG GTTTAATCCT GTTTATGATG TCAGTTATTA TAGAGTTAAT   99
LEPP S         --------AG ACCTTTGGTG GTTTAATCCT GTTTATGATG TCAGTTATTA TAGAGTTAAT   99
IA1894 S       ---------- ---------- ---------- ---------- ---------- ----------   44
ISU-1 S        ---------- ---------- ---------- ---------- ---------- ----------   62
PInd/89 S      ---------- ---------- ---------- ---------- ---------- ----------   62
86/137004 S    ---------- ---------- ---------- ---------- ---------- ----------   59
RM4 S          ---------- ---------- ---------- ---------- ---------- ----------   59

CHV S          AATAAAAATG GTACTACCGT AGTTTCCAAT TGCACTGATC AATGTGCTAG TTATGTGGCT  780
AR310 S        AATAAAAATG GTACTACCGT AGTTTCCAAT TGCACTGATC AATGTGCTAG TTATGTGGCT  159
LEPP S         AGTAAAAATG GTACTACCGG AGTTTCCAAT TGCACTGATC AATGTGCTAG TTATGTGGCT  159
IA1894 S       --TAAAAATG GTACTACCGT AGTTTCCAAT TGCACTGATC AATGTGCTAG TTATGTGGCT  102
ISU-1 S        ---------- ---------- ---TTCCAAT TGCACTGATC AATGTGCTAG TTATGTGGAT   99
Ind/89 S       ---------- ---------- ---TTCCAAT TGCACTGATC AATGTGCTAG TTATGTGGCT   99
86/137004 S    ---------- -TACTTCCGT AGTTTCCAAT TGCACTGATC AATGTGCTAG TTATGTGGCT  108
RM4 S          ---------- -TACTTCCGT AGTTTCCAAT TGCACTGATC AATGTGCTAG TTATGTGGCT  108
```

Figure 3. (continued).

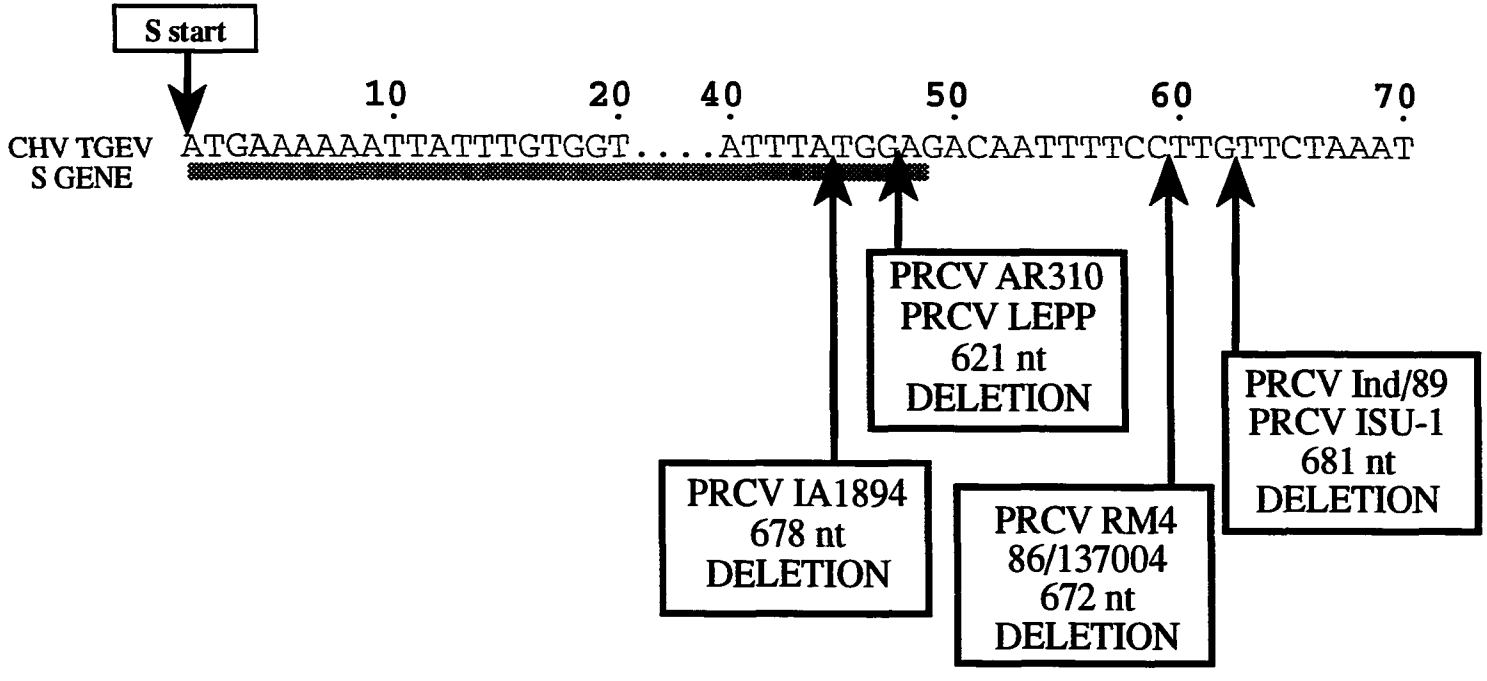


Figure 4. Schematic diagram showing the size and location of the S gene deletions of PRCV isolates. The underlined region represents the signal peptide region of the TGEV S gene as determined by Rasschaert and Laude (1987). The nucleotide sequences of the TGEV isolate CHV and of the PRCV isolates AR310, LEPP, IA1894, and ISU-1 were determined in this study. The nucleotide sequences of the other PRCV isolates were previously reported (Ind/89, Wesley et al., 1991; 86/137004, Britton et al., 1990; and RM4, Rasschaert et al., 1990). The nucleotide sequence located between positions 20 and 40 are not shown for clarity.

```
CHV S          MKKLFVVLVV MPLIYGDNFP CSKLTNRTIG NHWNLIETLL    40
AR310 S        MKTLFVVLVI MPLIYG---- ---------- ----------    16
LEPP S         MKTLFVVLVI MPLIYG---- ---------- ----------    16
IA1894 S       MKTLFVVLVI MPLIYKN--- ---------- ----------    17
ISU-1 S        MKTLFVVLVI MPLIYGDNFP C--------- ----------    21
Ind/89 S       MKTLFVVLVI MPLIYGDNFP C--------- ----------    21
86/137004 S    MKKLFVVLVV MPLIYGDKFP ---------- ----------    20
RM4 S          MKKLFVVLVV MPLIYGDKFP ---------- ----------    20
               ^^ ^^^^^^  ^^^^^
```

Figure 5. Alignment of the predicted amino acid residues from the 5' end of the S gene of the TGEV isolate CHV, and the PRCV isolates AR310, LEPP, IA1894, ISU-1, Ind/89, 86/137004, and RM4. The underlined amino acid residues indicate the signal peptide region at the 5' end of the S gene as determined by Rasschaert and Laude, 1987. Deleted amino acid residues are marked with (-) and identical amino acid residues are marked with (^). The PRCV isolates AR310 and LEPP had 207 amino acid residues deleted. The PRCV isolate IA1894 had 226 amino acid residues deleted. The PRCV isolates ISU-1 and Ind/89 both had 227 amino acid residues deleted. The PRCV isolates 86/137004 and RM4 both had 224 amino acid residues deleted The predicted amino acid residues were derived from the nucleotide sequences of the TGEV isolate CHV and of the PRCV isolates AR310, LEPP, IA1894, and ISU-1 determined in this study. The other amino acid residues presented in this table were previously reported (Ind/89, Wesley et al., 1991; 86/137004 PRCV, Page et al., 1991; and RM4 PRCV, Rasschaert et al., 1990).

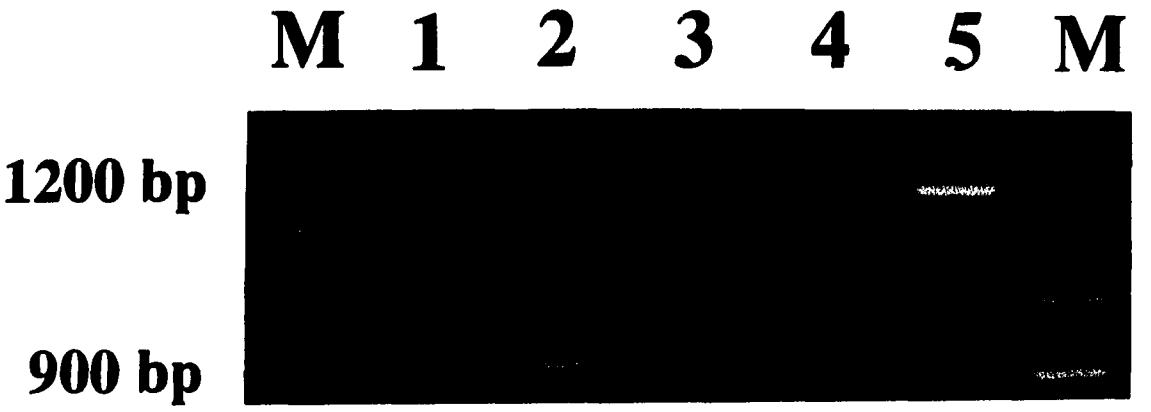
```
CHV S          LNYSSRLSPN SDVVLGDYFP TVQPWFNCIR NNSNDLYVTL    80
AR310 S        ---------- ---------- ---------- ----------    16
LEPP S         ---------- ---------- ---------- ----------    16
IA1894 S       ---------- ---------- ---------- ----------    17
ISU-1 S        ---------- ---------- ---------- ----------    21
Ind/89 S       ---------- ---------- ---------- ----------    21
86/137004 S    ---------- ---------- ---------- ----------    20
RM4 S          ---------- ---------- ---------- ----------    20

CHV S          QWSGTVTLGD MRATTLEAAG TLVDLWWFNP VYDVSYYRVN   240
AR310 S        ---------- ---------- ---DLWWFNP VYDVSYYRVN    33
LEPP S         ---------- ---------- ---DLWWFNP VYDVSYYRVN    33
IA1894 S       ---------- ---------- ---------- ----------    17
ISU-1 S        ---------- ---------- ---------- ----------    21
Ind/89 S       ---------- ---------- ---------- ----------    21
86/137004 S    ---------- ---------- ---------- ----------    20
RM4 S          ---------- ---------- ---------- ----------    20

CHV S          NKNGTTVVSN CTDQCASYVA     260
AR310 S        NKNGTTVVSN CTDQCASYVA      53
LEPP S         SKNGTTGVSN CTDQCASYVA      53
IA1894 S       ---GTTVVSN CTDQCASYVA      34
ISU-1 S        --------SN CTDQCASYVD      33
Ind/89 S       --------SN CTDQCASYVA      33
86/137004 S    ----TSVVSN CTDQCASYVA      36
RM4 S          ----TSVVSN CTDQCASYVA      36
                      ^^ ^^^^^^^^^^
```

Figure 5. (continued).

Figure 6. PCR amplification of the 3/3a and 3-1/3b gene region of TGEV and PRCV isolates. cDNA was amplified by PCR with primers 538 and 622 as described in text. Molecular weight markers (M), and PCR products from CHV TGEV (lane 1), ISU-1 (lane 2), AR310 (lane 3), IA1894 (lane 4), LEPP (lane 5) were separated on 2.0% agarose gel. Note that the PCR products from TGEV, AR310 and LEPP migrate at the same rate. The PCR product from IA1894 migrated slightly faster whereas the PCR product from ISU-1 migrated much faster.


M 1 2 3 4 5 M
1200 bp
900 bp

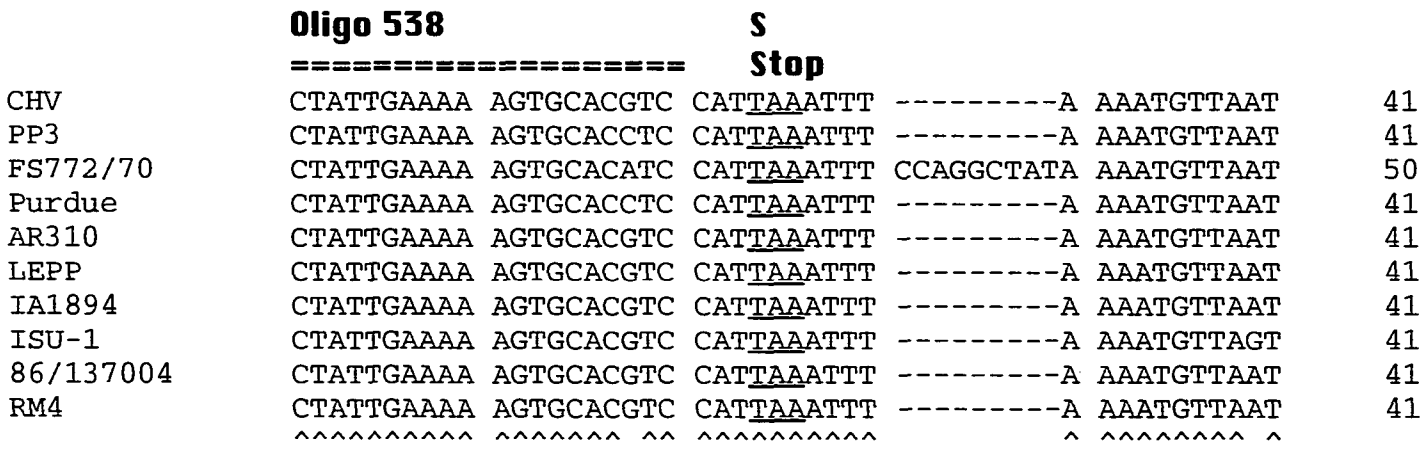


Figure 7. Comparison of the nucleotide sequence of the gene 3/3a and gene 3-1/3b regions of TGEV and PRCV isolates. Positions of the leader RNA binding site upstream of genes are marked with (*****). The positions of oligonucleotide primers 538, 118, 048, and 622 are marked with (=====). The start codons of the ORFs are marked with +1→, and the stop codons are underlined. Positions having identical nucleotides are marked with (^), and positions of deleted nucleotides are marked with (-----). The nucleotide sequences of the TGEV isolate CHV and of the PRCV isolates AR310, LEPP, IA1894, and ISU-1 were determined in this study. The other nucleotide sequences presented in this table were previously reported (PP3 (Miller) TGEV, Wesley et al., 1988; FS772/70 TGEV, Britton et al., 1989; Purdue TGEV, Kapke et al., 1988; 86/137004 PRCV, Page et al., 1991; and RM4 PRCV, Rasschaert et al., 1990).

```
CHV          TTTATTATCT GCTATAATAG CATTTGTT-- ---------- ----GTTAAG    75
PP3          TTTATTATCT GCTATAATAG CATTTGTT-- ---------- ----GTTAAG    75
FS772/70     TTTATTATCT GCTATAATAG CATTTGTT-- ---------- ----GTTAAG    84
Purdue       TCTATCATCT GCTATAATAG CAGTTGTTTC TGCTAGAGAA TTTTGTTAAG    91
AR310        T---TTATCT GCTATAATAG CATTTGTT-- ---------- ----ATTAAG    72
LEPP         T---TTATCT GCTATAATAG CATTTGTT-- ---------- ----ATTAAG    72
IA1894       T---TTATCT GCTATAATAG CATTTGTT-- ---------- ----ATTAAG    72
ISU-1        T---TTATCC GCTAT----- ---------- ---------- ----------    53
86/137004    T---TTATCT GCTATAATAT CATTTGTT-- ---------- ----GTTAAG    72
RM4          TTTATC-TCT GCTATAATAT CATTTGTT-- ---------- ----GTTAAG    74
             ^   ^  ^^  ^^^^^

                                      ******
CHV          GATGATGAAT AAAGTCCTTA AGAACTAAAC TTTCGAGTCA TTACAGGTCC   125
PP3          GATGATGAAT AAAGTCCTTA AGAACTAAAC TTTCGAGTCA TTACAGGTCC   125
FS772/70     GATGATGAAT AAAGTCCTTA AGAACTAAAC TTTCGAGTCA TTACAGGTCC   134
Purdue       GATGATGAAT AAAGTCTTTA AGAACTAAAC TTACGAGTCA TTACAGGTCC   141
AR310        GATGATGAAT AAAGTCCTTA AGAACTAAAC TTTCAGGTCA TTACAGGTCC   122
LEPP         GATGATGAAT AAAGTCCTTA AGAACTAAAC TTTCTGGTCA TTACAGGTCC   122
IA1894       GATGATGAAT AAAGTCCTTA AGAACTAAAC TTTCAGGTCA TTACAGGTCC   122
ISU-1        ---------- ---------- ---------- ---------- ----------    53
86/137004    GATGATGAAT AAAG------ -------AAC TTTCAAGTCA ----------    99
RM4          GATGATGAAT AAAG------ -------AAC TT-CAAGTCA ----------   100
```

Figure 7. (continued).

ORF 3

+1→

```
CHV          TGTATGGACA TTGTCAAATC CATTAATACA TCCGTAGATG CTGTACTTGA  175
PP3          TGTATGGACA TTGTCAAATC CATTAATACA TCCGTAGATG CTGTACTTGA  175
FS772/70     TGTATGGACA TTGTCAAATC CATTAATACA TCCGTAGATG CTGTACTTGA  184
Purdue       TGTATGGACA TTGTCAAATC CATTTACACA TCCGTAGATG CTGTACTTGA  191
AR310        TGTATGGACA TTGTCAAATC TATTAATACA TCCGTGGATG CTGTACTTGA  172
LEPP         TGTATGGACA TTGTCAAATC TATTAATACA TCCGTGGATG CTGTACTTGA  172
IA1894       TGTATGGACA TTGGCAAATC CATTATTACA TCCGTGGATG CTGTACTTGA  172
ISU-1        ---------- ---------- ---------- ---------- ----------   53
86/137004    ---------- --GTCAAATT TACTAATACA TCCGTGGACG TTGTACTTGA  137
RM4          ---------- --G-CAAATT TACTAATACA TCCGTGGACG TTGTACTTGG  137

CHV          CGAACTTGAT TGTGCATACT TTGCTGTAAC TCTTAAAGTA GAATTTAAGA  225
PP3          CGAACTTGAT TGTGCATACT TTGCTGTAAC TCTTAAAGTA GAATTTAAGA  225
FS772/70     CGAACTTGAT TGTGCATACT TTGCTGTAAC TCTTAAAGTA GAATTTAAGA  234
Purdue       CGAACTTGAT TGTGCATACT TTGCTGTAAC TCTTAAAGTA GAATTTAAGA  241
AR310        CGAACTTGAT TGTGCATACT TCGCTGTTAC TCTTAAAGTA GAATTTAAGA  222
LEPP         CGAACTTGAT TGTGCATACT TCGCTGTTAC TCTTAAAGTA GAATTTAAGA  222
IA1894       CGAACTTGAT TGTGCATACT TCGCTGTAAC TCTTAAAGTA GAATTTAAGA  222
ISU-1        ---------- ---------- ---------- ---------- ---------A   54
86/137004    CGAACTTGAT TGTGTATACT TTGCTGTAAC CCTTAAAGTA GAATTTAAGA  187
RM4          CGAACTTGAT TGTGTATACT TTACTGTAAC CCTTAAAGTA GAATTTAAGA  187
                                                                  ^
```

Figure 7. (continued).

```
CHV          CTGGTAAATT ACTTGTGTGT ATAGGTTTTG GTGACACACT TCTTGCGGCT   275
PP3          CTGGTAAATT ACTTGTGTGT ATAGGTTTTG GTGACACACT TCTTGCGGCT   275
FS772/70     CTGGTAAATT ACTTGTGTGT ATAGGTTTTG GTGACACACT TCTTGCGGCT   284
Purdue       CTGGTAAATT ACTTGTGTGT ATAGGTTTTG GTGACACACT TCTTGCTGCT   291
AR310        CTGGTAAATT ACTTGTGTGT ATAGGTTTTG GTGACACACT TCTTGCGGCT   272
LEPP         CTGGTAAATT ACTTGTGTGT ATAGGTTTTG GTGACACACT TCTTGCGGCT   272
IA1894       CTGGTAAATT ACTTGTGTGT ATAGGTTTTG GTGACACACT TCTTGCGGCT   272
ISU-1        CTGGTAAATT ACTTGTGTGT ATAGGTTTTG GTGACACACT TCTTGCGGCT   104
86/137004    CTTGTAAATT ACTTGTGTGC ATAGGTTTTG GTGACATACT TCTTGCGGCT   237
RM4          CTTGTAAATT ACTTGTGTGC ATAGGTTTTG GTGACATACT TCTTGCGGCT   237
             ^^ ^^^^^^^ ^^^^^^^^^  ^^^^^^^^^^ ^^^^^^ ^^^ ^^^^^^^^^^
```

Oligo 118

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                                                 =========
CHV          AGGGATAAAG CATATGCTAA GCTTGGTCTC TCCATTATTG AAGAAGT---   322
PP3          AGGGATAAAG CATATGCTAA GCTTGGTCTC TCCATTATTG AAGAAGT---   322
FS772/70     AGGGATAAAG CATATGCTAA GCTTGGTCTC TCCACTATTT AAGAAGT---   331
Purdue       AAGGATAAAG CATATGCTAA GCTTGGTCTC TCCATTATTG AAGAAGTCAA   341
AR310        AGGGATAAAG CATATGCTAA GCTTGGTCTC GCCACTATTG AAGAAGT---   319
LEPP         AGGGATAAAG CATATGCTAA GCTTGGTCTC GCCACTATTG AAGAAGT---   319
IA1894       AGGGGTAAAG CATAT----- ---------- --------TG AAGAAGT---   296
ISU-1        AGGGATAAAG CATA-----A GCTTGGTCTC GCCACTATTG AAGAAGT---   146
86/137004    A--------- ---------- ---------- -------TTG AAGAAGT---   248
RM4          A--------- ---------- ---------- -------TTG AAGAAGT---   248
             ^                                          ^  ^^^^^^^
```

Figure 7. (continued).

```
                                                    Oligo 118
                                                    ==========
CHV          ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   346
PP3          ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   346
FS772/70     ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   355
Purdue       TAGTCATATA GTTGTTTAAT ATCATTAAAC ACACAAAACC CAAAGCATTA   391
AR310        ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   343
LEPP         ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   343
IA1894       ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   320
ISU-1        ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   170
86/137004    ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   272
RM4          ---------- ---------- ------AAAC ACACAAAATC CAAAGCATTA   272
                                    ^^^^ ^^^^^^^^ ^ ^^^^^^^^^^

                                                                *****
CHV          AGTGTTACAA AACAATTAAA GAGAGATTAT AGAAAAACTG TCATTCTAAA   396
PP3          AGTGTTACAA AACAATTAAA GAGAGATTAT AGAAAAACTG TCATTCTAAA   396
FS772/70     AGTGTTACAA AACAATTAAA GAGAGATTAT AGAAAAACTG TCATTCTAAA   405
Purdue       AGTGTTACAA AACAATTAAA GAGAGATTAT AGAAAAACTG TCATTCTAAA   441
AR310        AGTGTTACAA AACAATTAAA GAGAGATTAT AGAAAAACTG TCATTCTAAA   393
LEPP         AGTGTTACAA AACAATTAAA GAGAGATTAT AGAAAAACTG TCATTCTAAA   393
IA1894       AGTGTTACAA AACAATTAAA GAGAGATTGT AGAAAAACTG TCATTCTAA-   369
ISU-1        AGTGTTACAA AACAATTAAA GAGAGATTAT AGAAAAACTG TCATTCTAAA   220
86/137004    AGTGTTACAA AACAATTAAA GAGAGACTAT AGAAAAACT- -CGAACTAAA   320
RM4          AGTGTTACAA AACAATTAAA GAGAGACTAT AGAAAAACT- -CGACCTAAA   320
             ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^ ^^^ ^^^^^^^^^   ^   ^^^^
```

Figure 7. (continued).

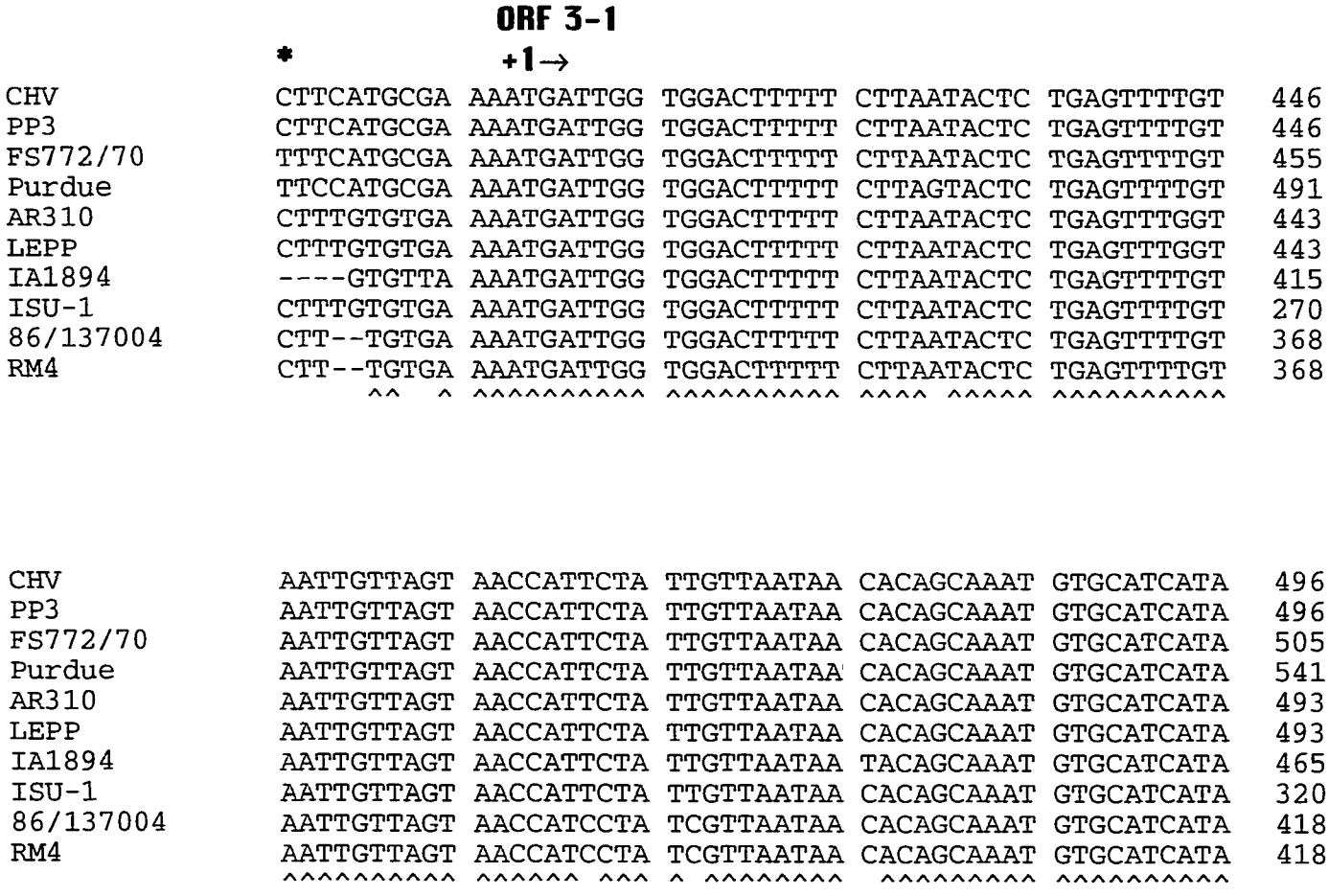

Figure 7. (continued).

```
CHV          TAAAACAAGA ACGTGTTATA GTACAACAGC ATCAGGTTGT TAGTGCTAGA   546
PP3          TAAAACAAGA ACGTGTTATA GTACAACAGC ATCAGGTTGT TAGTGCTAGA   546
FS772/70     TACAACAAGA ACGTGTTATA GTACAACAGC ATCAGGTTGT TAGTGCTATA   555
Purdue       TACAACAAGA ACGTGTTATA GTACAACAGC ATCAGGTTGT TAGTGCTAGA   591
AR310        CACAACAAGA CCGTGTTATA GTACAACAGC ATCAGGTTGT TAGTGCTAGA   543
LEPP         CACAACAAGA CCGTGTTATA GTACAACAGC ATCAGGTTGT TAGTGCTAGA   543
IA1894       CACAACAAGA CCGTGTTATA GTACAACATC ATCAGGTTAT TAGTGCTAGA   515
ISU-1        CACAACAAG- ---------- ---------- ---------- ----------   329
86/137004    TACAACAAGA ACGTGTTATA GTACAACAGC ACCATGTTGT TAGTGCTAGA   468
RM4          TACAACAAGA ACGTGTTATA GTACAACAGC ACCATGTTGT TAGTGCTAGA   468
              ^ ^^^^^^

CHV          ACACAAAATT ATTACCCAGA GTTCAGCATC GCTGTACTTT TTGTATCTTT   596
PP3          ACACAAAATT ATTACCCAGA GTTCAGCATC GCTGTACTTT TTGTATCTTT   596
FS772/70     ACACAAAACT ATTACCCAGA GTTCAGCATT GCTGTACTTT TTGTATCTTT   605
Purdue       ACACAAAACT ATTACCCAGA GTTCAGCATC GCTGTACCCT TTGTATCTTT   641
AR310        ACACAAAATT ATTACCCAGA GTTCAGCATC GCTGTAC-TT TTGTATCTTT   592
LEPP         ACACAAAATT ATTACCCAGA GTTCAGCATC GCTGTAC-TT TTGTATCTTT   592
IA1894       GCACAAAATT ATTATCCAGA GTTCAGCATC GCTGTACTTT TTGTATCTTT   565
ISU-1        ---------- ---------- ---------- ---------- ----------   329
86/137004    ACACAAAACT ATTACCCAGA GTTCAGCATC GCTGTACTTT TTGTATCTTT   518
RM4          ACACAAAACT ATTACCCAGA GTTCAGCATC GCTGTACTTT TTGTATCTTT   518
```

Figure 7. (continued).

```
CHV          TCTAGCTTTG TACCGTAGTA CAAACTTTAA GACGTGTGTC GGCATCTTAA   646
PP3          TCTAGCTTTG TACCGTAGTA CAAACTTTAA GACGTGTGTC GGCATCTTAA   646
FS772/70     TCTAGCTTTG TACCGCAGTA CAAACTTTAA GACGTGTGTC GGCATCTTAA   655
Purdue       TCTAGCTTTG TACCGTAGTA CAAACTTTAA GACGTGTGTC GGCATCTTAA   691
AR310        CCTAGCTTTG TACCGTAGTA CAAACTTTAA GACGTGTGTC GGTATCTTAA   642
LEPP         CCTAGCTTTG TACCGTAGTA CAAACTTTAA GACGTGTGTC GGTATCTTAA   642
IA1894       TCTAGCTTTG TACCGCAGTC CAAACTTTAA GACGTGTGTC GGTATCTTAA   615
ISU-1        ---------- ---------- ------TTAA GACGTGTGTC GGTATCTTAA   353
86/137004    TCTAGCTTTG TACCGTAGTA CAAACTTTAA GACGTGTGTC GGCATCTTAA   568
RM4          TCTAGCTTTG TACCGTAGTA CAAACTTTAA GACGTGTGTC GGCATCTTAA   568
                                   ^^^^ ^^^^^^^^^^ ^^ ^^^^^^^
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Oligo 048

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                                   ======================
CHV          TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGCATAT   696
PP3          TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGCATAT   696
FS772/70     TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGCATAT   705
Purdue       TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGCATAT   741
AR310        TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGTATAT   692
LEPP         TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGTATAT   692
IA1894       TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGTATAT   665
ISU-1        TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGTATAT   403
86/137004    TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGCATAC   618
RM4          TGTTTAAGAT TTTATCAATG ACACTTTTAG GACCTATGCT TATAGCATAC   618
             ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^ ^^^
```


Figure 7. (continued).

```
CHV          GGTTACTACA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   746
PP3          GGTTACTACA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   746
FS772/70     GGTTACTATA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   755
Purdue       GGTTACTACA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   791
AR310        GGTTACTACA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   742
LEPP         GGTTACTACA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   742
IA1894       GGTTACTACA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   715
ISU-1        GGTTACTACA TTGATGGCAT TGTTACAACA ACTTTCTTAT CTTTAAGATT   453
86/137004    GGTTACTACA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   668
RM4          GGTTACTACA TTGATGGCAT TGTTACAACA ACTGTCTTAT CTTTAAGATT   668
             ^^^^^^^^^ ^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^

CHV          TGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAAGTTATTT   796
PP3          TGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   796
FS772/70     CGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   805
Purdue       TGTCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   841
AR310        CGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   792
LEPP         CGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   792
IA1894       CGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   765
ISU-1        CGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   503
86/137004    CGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   718
RM4          CGCCTACTTA GCATACTTTT GGTATGTTAA TAGTAGGTTT GAATTTATTT   718
              ^ ^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^
```

Figure 7. (continued).

```
CHV          TATACAATAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   846
PP3          TATACAATAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   846
FS772/70     TATATAATAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   855
Purdue       TATACAATAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   891
AR310        TATACAACAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   842
LEPP         TATACAACAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   842
IA1894       TATACAACAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   815
ISU-1        TATACAACAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   553
86/137004    TATACAATAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   768
RM4          TATACAATAC AACGACACTC ATGTTTGTAC ATGGCAGAGC TGCACCGTTT   768
             ^^^^ ^^ ^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^

CHV          AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTGTATG GTGGCATAAA   896
PP3          AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTGTATG GTGGCATAAA   896
FS772/70     AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTGTATG GTGGCATAAA   905
Purdue       ATGAGAAGTT CTCACAGCTC TATTTATGTC ACATTGTATG GTGGCATAAA   941
AR310        AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTATATG GTGGCATAAA   892
LEPP         AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTATATG GTGGCATAAA   892
IA1894       AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTGTATG GTGGCATAAA   865
ISU-1        AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTATATG GTGGCATAAA   603
86/137004    AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTGTATG GTGGCATAAA   818
RM4          AAGAGAAGTT CTCACAGCTC TATTTATGTC ACATTGTATG GTGGCATAAA   818
             ^ ^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^ ^^^^ ^^^^^^^^^^
```

Figure 7. (continued).

```
CHV          TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   946
PP3          TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   946
FS772/70     TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   955
Purdue       TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   991
AR310        TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   942
LEPP         TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   942
IA1894       TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   915
ISU-1        TTATATGTTT GTGAATGACT TCATGTTGCA TTTTGTAGAC CCTATGCTTG   653
86/137004    TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   868
RM4          TTATATGTTT GTGAATGACC TCACGTTGCA TTTTGTAGAC CCTATGCTTG   868
             ^^^^^^^^^^ ^^^^^^^^^^  ^^^ ^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^

CHV          TAAGCATAGC AATACGTGGC TTAGCTCATG CTGATCTAAC TGTAGTTAGA    996
PP3          TAAGCATAGC AATACGTGGC TTAGCTCATG CTGATCTAAC TGTAGTTAGA    996
FS772/70     TAAGCATAGC AATACGTGGC TTAGCTCATG CTGATCTAAC TGTAGTTAGA   1005
Purdue       TAAGCATAGC AATACGTGGC TTAGCTCATG CTGATCTAAC TGTAGTTAGA   1041
AR310        TAAGCATAGC AATACGTGGC TTAACTCATG CTGATCTAAC TGTAGTTAGA    992
LEPP         TAAGCATAGC AATACGTGGC TTAACTCATG CTGATCTAAC TGTAGTTAGA    992
IA1894       TAAGCATAGC AATACGTGGC TTAGCTCATG CTGATCTAAC TGTAGTTAGA    965
ISU-1        TAAGCATAGC AATACGTGGC TTAACTCATG CTGATCTAAC TGTAGTTAGA    703
86/137004    TAAGCATAGC AATACGTGGC TTAGCTCATG CTGATCTAAC TGTAGTTAGA    918
RM4          TAAGCATAGC AATACGTGGC TTAGCTCATG CTGATCTAAC TGTAGTTAGA    918
             ^^^^^^^^^^ ^^^^^^^^^^ ^^^ ^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^
```


Figure 7. (continued).

```
CHV          GCAGTTGAAC TTCTCAATGG TGATTTTATT TATGTATTTT CACAGGAGCC  1046
PP3          GCAGTTGAAC TTCTCAATGG TGATTTTATT TATGTATTTT CACAGGAGCC  1046
FS772/70     GCAGTTGAAC TTCTCAATGG TGATTTTATT TATGTATTTT CACAGGAGCC  1055
Purdue       GCAGTTGAAC TTCTCAATGG TGATTTTATT TATGTATTTT CACAGGAGCC  1091
AR310        GCAGTTGAAC TTCTCAATGG TGATTTTATT TATATATTTT CACAGGAGCC  1042
LEPP         GCAGTTGAAC TTCTCAATGG TGATTTTATT TATATATTTT CACAGGAGCC  1042
IA1894       GCAGTTGAAC TTCTCAATGG TGATTTTATT TATATATTTT CACAGGATTC  1015
ISU-1        GCAGTTGAAC TTCTCAATGG TGATTTTATT TATATATTTT CACAGGAGCC   753
86/137004    GCAGTTGAAC TTCTCAATGG TGATTTTATT TATGTATTTT CACAGGAGCC   968
RM4          GCAGTTGAAC TTCTCAATGG TGATTTTATT TATGTATTTT CACAGGAGCC   968
             ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^ ^^^^^^ ^^^^^^^  ^

                                                      ******
CHV          CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA  1096
PP3          CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA  1096
FS772/70     CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA  1105
Purdue       CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA  1141
AR310        CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA  1092
LEPP         CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA  1092
IA1894       TGTAGTTGGT GTTTACAATG CAGCCTTTTC TCAGGCAGTT CTAAACGAAA  1065
ISU-1        CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA   803
86/137004    CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA  1018
RM4          CGTAGTCGGT GTTTACAATG CAGCCTTTTC TCAGGCGGTT CTAAACGAAA  1018
              ^^^^^ ^^^ ^^^^^^^^^^ ^^^^^^^^^^ ^^^^^^ ^^^ ^^^^^^^^^^
```

Figure 7. (continued).

```
                                                     ORF 4       ORF 3-1
                                                      +1→        Stop
CHV          TTGACTTAAA AGAAGAAGAA GAAGACCGTA CCTATGACGT TTCCTAGGGC  1146
PP3          TTGACTTAAA AGAAGAAGAA GAAGACCGTA CCTATGACGT TTCCTAGGGC  1146
FS772/70     TTGACTTAAA AGAAGAAGAG GGAGACCGTA CCTATGACGT TTCCTAGGGC  1155
Purdue       TTGACTTAAA AGAAGAAGAA GAAGACCATA CCTATGACGT TTCCTAGGGC  1191
AR310        TTGACTTAAA AGAAGAAGAG GGAGACCGTA CCTATGACGT TTCCTAGGGC  1142
LEPP         TTGACTTAAA AGAAGAAGAG GGAGACCGTA CCTATGACGT TTCCTAGGGC  1142
IA1894       TTGACTTAAA AGAAGAAGAG GGAGACCGTA CCTATGACGT TTCCTAGGGC  1115
ISU-1        TTGACTTAAA AGAAGAAGAG GGAGACCGTA CCTATGACGT TTCCTAGGGC   853
86/137004    TTGACTTAAA AGAAGAAGAG GGAGACCGTA CCTATGACGT TTCCTAGGGC  1068
RM4          TTGACTTAAA AGAAGAAGAG GGAGACCGTA CCTATGACGT TTCCTAGGGC  1068
             ^^^^^^^^^^ ^^^^^^^^^  ^ ^^^^^ ^^ ^^^^^^^^^^ ^^^^^^^^^^
```



```
CHV          ATTGACTGTC ATAGATGACA ATGGAATGGT CATTAGCATC ATT         1189
PP3          ATTGACTGTC ATAGATGACA ATGGAATGGT CATTAGCATC ATT         1189
FS772/70     ATTGACTGTC ATAGATGACA ATGGACTGGT CATTAGCATC ATT         1198
Purdue       ATTGACTGTC ATAGATGACA ATGGAATGGT CATTAACATC ATT         1234
AR310        ATTGACTGTC ATAGATGACA ATGGAATGGT CATTAGCATC ATT         1185
LEPP         ATTGACTGTC ATAGATGACA ATGGAATGGT CATTAGCATC ATT         1185
IA1894       ATTGACTGTC ATAGATGACA ATGGAATGGT CATTAGCATC ATT         1158
ISU-1        ATTGACTGTC ATAGATGATA ATGGAATGGT CATTAGCATC ATT          896
86/137004    ATTGACTGTC ATAGATGACA ACGGAATGGT CATTAGCATC ATT         1111
RM4          ATTGACTGTC ATAGATGACA ACGGAATGGT CATTAGCATC ATT         1111
             ^^^^^^^^^^ ^^^^^^^^ ^ ^^^^=====================
                                              Oligo 622
```

Figure 7. (continued).

```
CHV 3           MDIVKSINTS VDAVLDELDC AYFAVTLKVE FKTGKLLVCI      40
PP3 3           .......... .......... .......... ..........      40
FS772/70 3a     .......... .......... .......... ..........      40
Purdue 3a       .......Y.. .......... .......... ..........      40
AR310 3         .......... .......... .......... ..........      40
LEPP 3          .......... .......... .......... ..........      40
IA1894 3a       ...G...I.. .......... .......... ..........      40


CHV 3           GFGDTLLAAR DKAYAKLGLS IIEEVNTQNP KH       72
PP3 3           .......... .......... .......... ..       72
FS772/70 3a     .......... .......... T.                   62
Purdue 3a       ........K. .......... ......SHIV V        71
AR310 3         .......... .........A T......... ..       72
LEPP 3          .......... .........A T......... ..       72
IA1894 3a       .......... G...Y                           55
```


Figure 8. Alignment of the predicted amino acid residues from the 3/3a gene of TGEV and PRCV isolates. Identical amino acid residues are marked with (.). The TGEV isolates CHV and PP3 have identical amino acid homology and are both 72 amino acid residues long. The TGEV isolate FS772/70 is 62 amino acid residues long. The Purdue TGEV isolate has an 3a protein 71 amino acid residues in length and the last six amino acid residues are changed from that of CHV TGEV. The PRCV isolates AR310 and LEPP have identical 3 proteins that are 72 amino acid residues in length and differ from that of CHV TGEV by two amino acid residues. The PRCV isolate IA1894 has a truncated 3a protein of 53 amino acid residues in length due to a 23 nucleotide deletion in the coding sequence of the 3a gene.

```
CHV 3-1          MIGGLFLNTL SFVIVSNHSI VNNTANVHHI KQERVIVQQH QVVSARTQNY    50
PP3 3-1          .......... .......... .......... .......... ..........    50
Purdue 3-1b      .......S.. .......... .......... Q......... ..........    50
AR310 3-1        .......... .L........ .........T Q.D....... ..........    50
LEPP 3-1         .......... .L........ .........T Q.D....... ..........    50
FS772/70 3-1b    .......... .......... .......... Q......... .....I....    50
ISU-1 3-1        .......... .......... .........T Q.--------- ----------    32
IA1894 3-1b      .......... .......... .........T Q.D.....H. ..I...A...    50
86/137004 3-1    .......... .........P. .......... Q......... H.........    50
RM4 3-1          .......... .........P. .......... Q......... H.........    50
```


Figure 9. Alignment of the predicted amino acid residues from the 3-1/3b genes of TGEV and PRCV isolates. Identical amino acid residues are marked with (.). The TGEV isolates CHV, PP3, Purdue, FS772/70, and the PRCV isolates IA1894, 86/137004, and RM4 all have nearly identical 3-1/3b proteins of 244 amino acid residues in length. The PRCV isolates AR310 and LEPP both have truncated 3-1 proteins of 64 amino acid residues in length due to an one nucleotide deletion that results in a frameshift in the coding region of the 3-1 gene. The PRCV isolate ISU-1 has a truncated 3-1 protein of 205 amino acid residues due to a 117 nucleotide inframe deletion in the coding region of the 3-1 gene.

```
CHV 3-1          YPEFSIAVLF VSFLALYRST NFKTCVGILM FKILSMTLLG PMLIAYGYYI   100
PP3 3-1          .......... .......... .......... .......... ..........   100
Purdue 3-1b      ........P. .......... .......... .......... ..........   100
AR310 3-1        .........L YLSS                                           64
LEPP 3-1         .........L YLSS                                           64
FS772/70 3-1b    .......... .......... .......... .......... ..........   100
ISU-1 3-1        ---------- ---------- -V........ .......... ....V.....    61
IA1894 3-1b      .......... .........P .......... .......... ....V.....   100
86/137004 3-1    .......... .......... .......... .......... ..........   100
RM4 3-1          .......... .......... .......... .......... ..........   100

CHV 3-1          DGIVTTTVLS LRFAYLAYFW YVNSRFEVIL YNTTTLMFVH GRAAPFKRSS   150
PP3 3-1          .......... .......... .......F.. .......... ..........   150
Purdue 3-1b      .......... ...V...... .......F.. .......... ......M...   150
AR310 3-1                                                                  64
LEPP 3-1                                                                   64
FS772/70 3-1b    .......... .......... .......F.. .......... ..........   150
ISU-1 3-1        .......F.. .......... .......F.. .......... ..........   111
IA1894 3-1b      .......... .......... .......F.. .......... ..........   150
86/137004 3-1    .......... .......... .......F.. .......... ..........   150
RM4 3-1          .......... .......... .......F.. .......... ..........   150
```

Figure 9. (continued).

```
CHV 3-1          HSSIYVTLYG GINYMFVNDL TLHFVDPMLV SIAIRGLAHA DLTVVRAVEL   200
PP3 3-1          .......... .......... .......... .......... ..........   200
Purdue 3-1b      .......... .......... .......... .......... ..........   200
AR310 3-1                                                                  64
LEPP 3-1                                                                   64
FS772/70 3-1b    .......... .......... .......... .......... ..........   200
ISU-1 3-1        .......... .........F M......... .......T.. ..........   161
IA1894 3-1b      .......... .......... .......... .......... ..........   200
86/137004 3-1    .......... .......... .......... .......... ..........   200
RM4 3-1          .......... .......... .......... .......... ..........   200
```

```
CHV 3-1          LNGDFIYVFS QEPVVGVYNA AFSQAVLNEI DLKEEEEDRT YDVS    244
PP3 3-1          .......... .......... .......... .......... ....    244
Purdue 3-1b      .......... .......... .......... ........H. ....    244
AR310 3-1                                                            64
LEPP 3-1                                                             64
FS772/70 3-1b    .......... .......... .......... ......G... ....    244
ISU-1 3-1        .......I.. .......... .......... ......G... ....    205
IA1894 3-1b      .......I.. .DS....... .......... ......G... ....    244
86/137004 3-1    .......... .......... .......... ......G... ....    244
RM4 3-1          .......... .......... .......... ......G... ....    244
```

Figure 9. (continued).

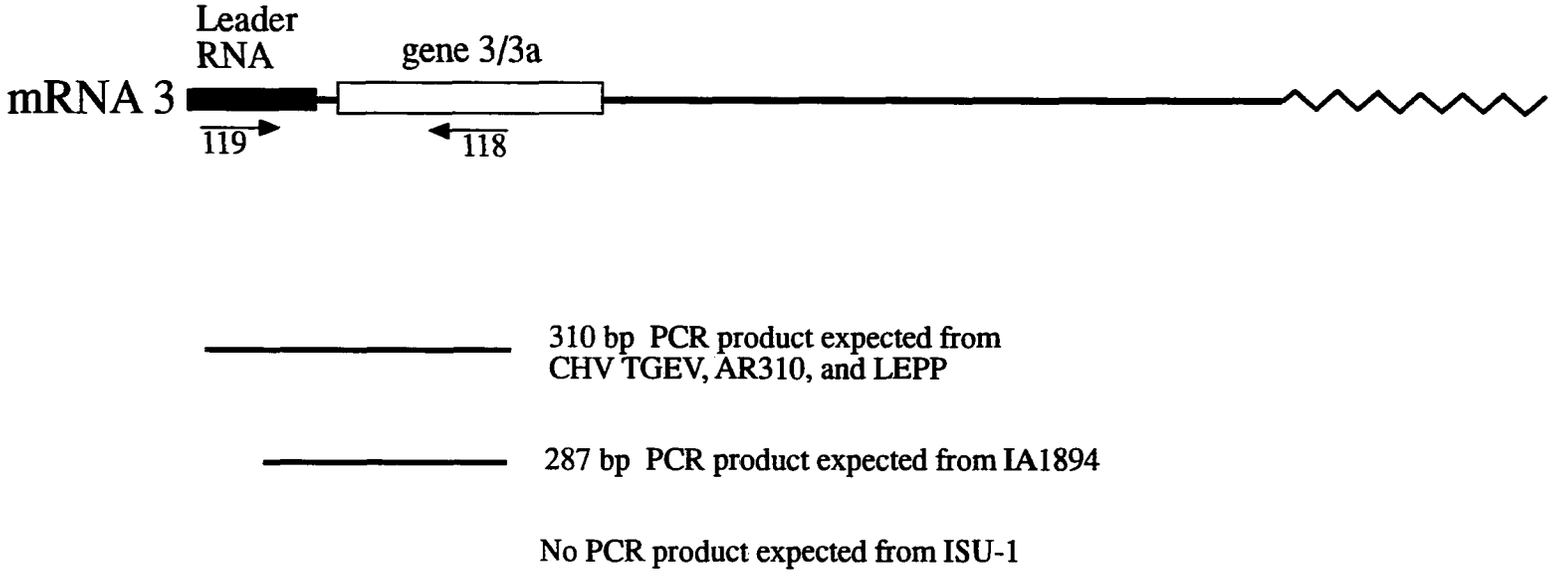


Figure 10. Schematic diagram of the PCR products expected from amplification of mRNA 3 with the primers 119 and 118 using conditions described in the text. The TGEV isolate CHV and the PRCV isolates AR310 and LEPP would be expected to yield a PCR product of 310 bp since these isolates have a CTAAAC leader RNA binding site preceding gene 3. The PRCV isolate IA1894 would be expected to yield a smaller PCR product of 287 bp since this isolate has a CTAAAC leader RNA binding site preceding gene 3a and also has a 23 nucleotide deletion present in the coding region of gene 3a. The PRCV isolate ISU-1 would be expected to have no PCR product since the CTAAAC leader RNA binding site preceding gene 3 is removed due to a 168 nucleotide deletion.

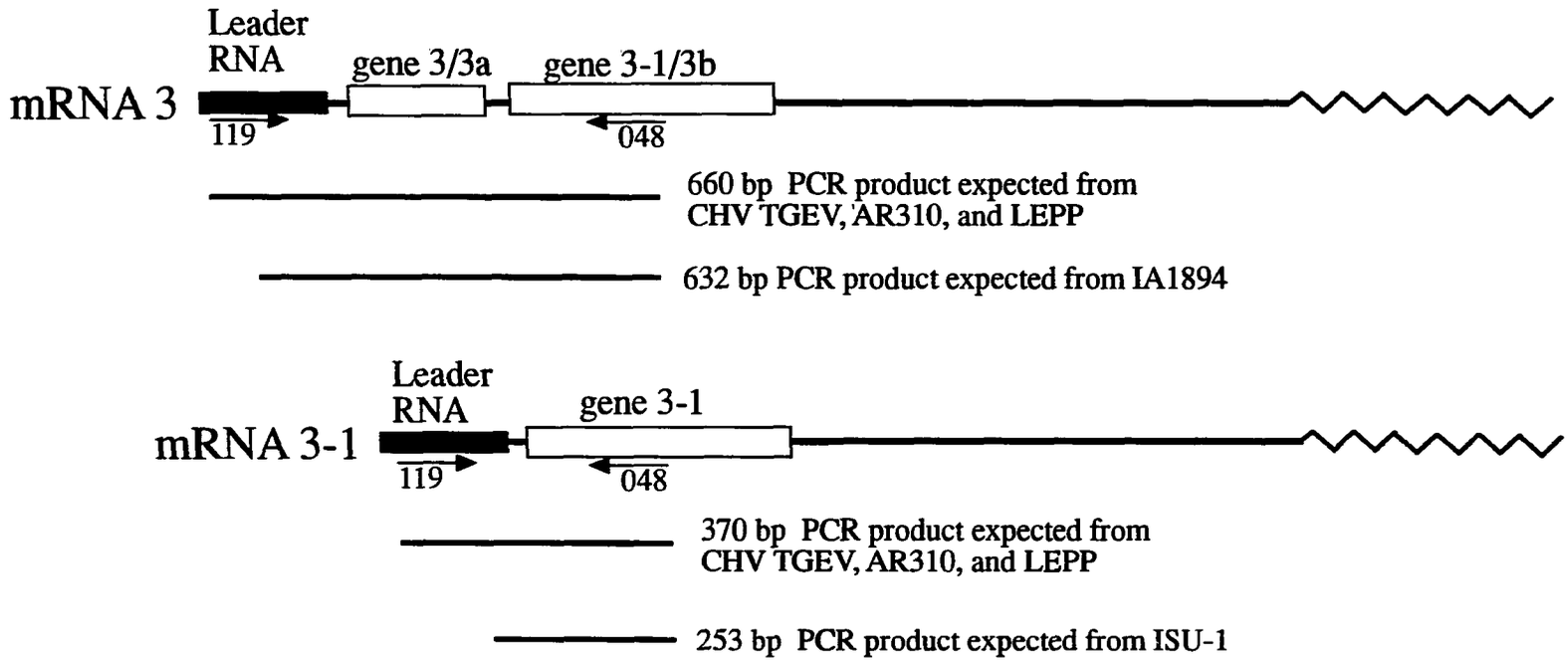


Figure 11. Schematic diagram of the PCR products expected from amplification of mRNA 3 and/or mRNA 3-1 with the primers 119 and 048 using conditions described in the text. The TGEV isolate CHV and the PRCV isolates AR310 and LEPP would be expected to yield two PCR products of 660 bp and 370 bp because these isolates all produce mRNA 3 and mRNA 3-1 since there is a CTAAAC leader RNA binding site preceding both the 3 and 3-1 genes. The PRCV isolate IA1894 would be expected to yield a single PCR product of 632 bp since this isolate has a CTAAAC leader RNA binding site preceding the 3a gene but not preceding the 3b gene. The PCR product from the PRCV isolate IA1894 is smaller because of a 23 nucleotide deletion present in the coding region of gene 3a. The PRCV isolate ISU-1 would be expected to have a single PCR product of 253 bp since there is a CTAAAC leader RNA binding site only preceding the 3-1 gene as the CTAAAC leader RNA binding site preceding gene 3 is removed due to a 168 nucleotide deletion. The PCR product from the PRCV isolate ISU-1 is smaller because of a 117 nucleotide inframe deletion within the coding region of the 3-1 gene.

Figure 12. PCR amplification of the 5' end of the 3/3a gene of TGEV and PRCV isolates. cDNA was amplified by PCR with primers 119 and 118 as described in text. Molecular weight markers (M), and PCR products from CHV TGEV (lane 1), ISU-1 (lane 2), AR310 (lane 3), IA1894 (lane 4), LEPP (lane 5) were separated on 1.5% agarose gel. PCR products were generated for CHV TGEV and the PRCV isolates AR310, LEPP, and IA1894 which confirms the joining of the leader RNA to the CTAAAC leader RNA binding site preceding the 3/3a gene. Note that the PCR products of 310 bp migrated at the same rate for CHV TGEV, AR310 and LEPP. The PCR product of 287 bp from IA1894 migrated slightly faster due to a 23 nucleotide deletion in the coding region of gene 3a. No PCR product was evident from the PRCV isolate ISU-1 due to the CTAAAC leader RNA binding site preceding the 3 gene being removed by a 168 nucleotide deletion.

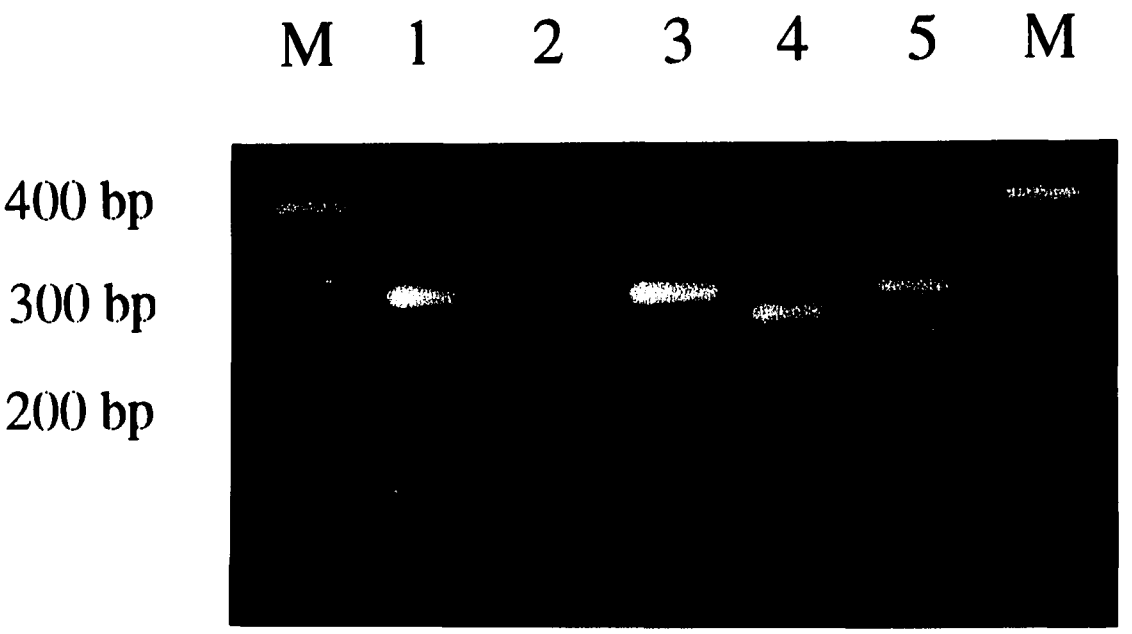
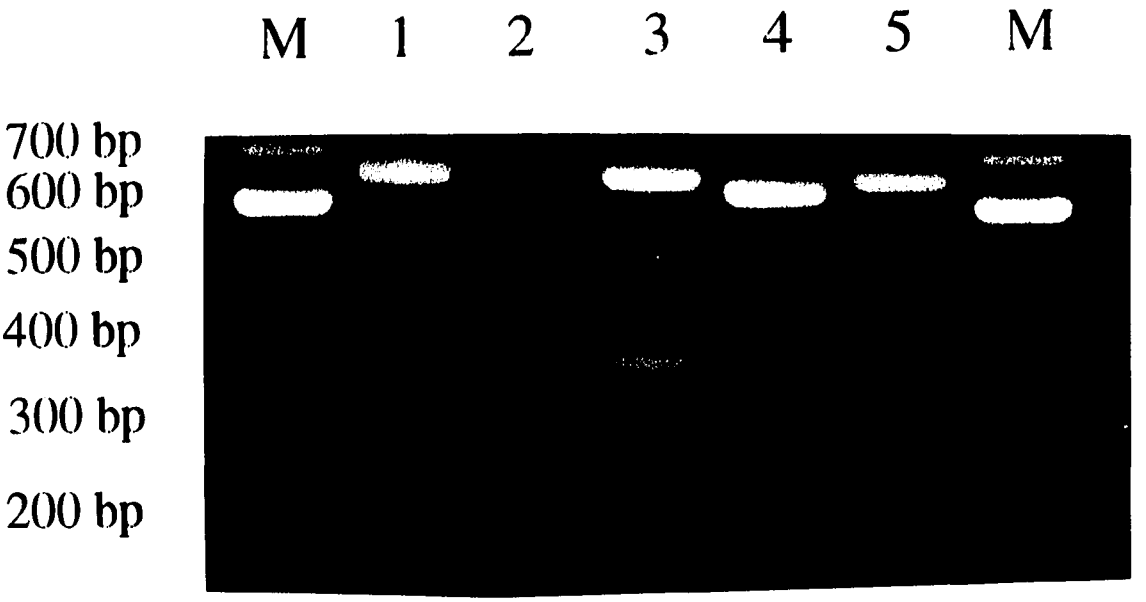

M 1 2 3 4 5 M
400 bp
300 bp
200 bp

Figure 13. PCR amplification of the 5' end of the 3-1/3b gene of TGEV and PRCV isolates. cDNA was amplified by PCR with primers 119 and 048 as described in text. Molecular weight markers (M), and PCR products from CHV TGEV (lane 1), ISU-1 (lane 2), AR310 (lane 3), IA1894 (lane 4), LEPP (lane 5) were separated on 1.5% agarose gel. Note that two PCR products of 660 and 370 bp were amplified from CHV TGEV, AR310 and LEPP indicating that the leader sequence had bound to the CTAAAC leader RNA binding site preceding the 3 and 3-1 genes. The PRCV isolate IA1894 had only a single PCR product of 632 bp evident, which is consistent with the 23 nucleotide deletion in the 3a gene and with the leader sequence not binding to the CTAAAC leader RNA binding site preceding the 3b gene due to a 5 nucleotide deletion removing the last two nucleotides of the CTAAAC sequence. The PRCV isolate ISU-1 had only a single PCR product of 253 bp evident, which is consistent with there being a CTAAAC leader RNA binding site preceding the 3-1 gene and a 117 nucleotide inframe deletion present in the coding region of gene 3-1, and the loss of the CTAAAC leader RNA binding site preceding the 3 gene due to the 168 nucleotide deletion present in the 3 gene region.


M
1
2
3
4
5
M
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp

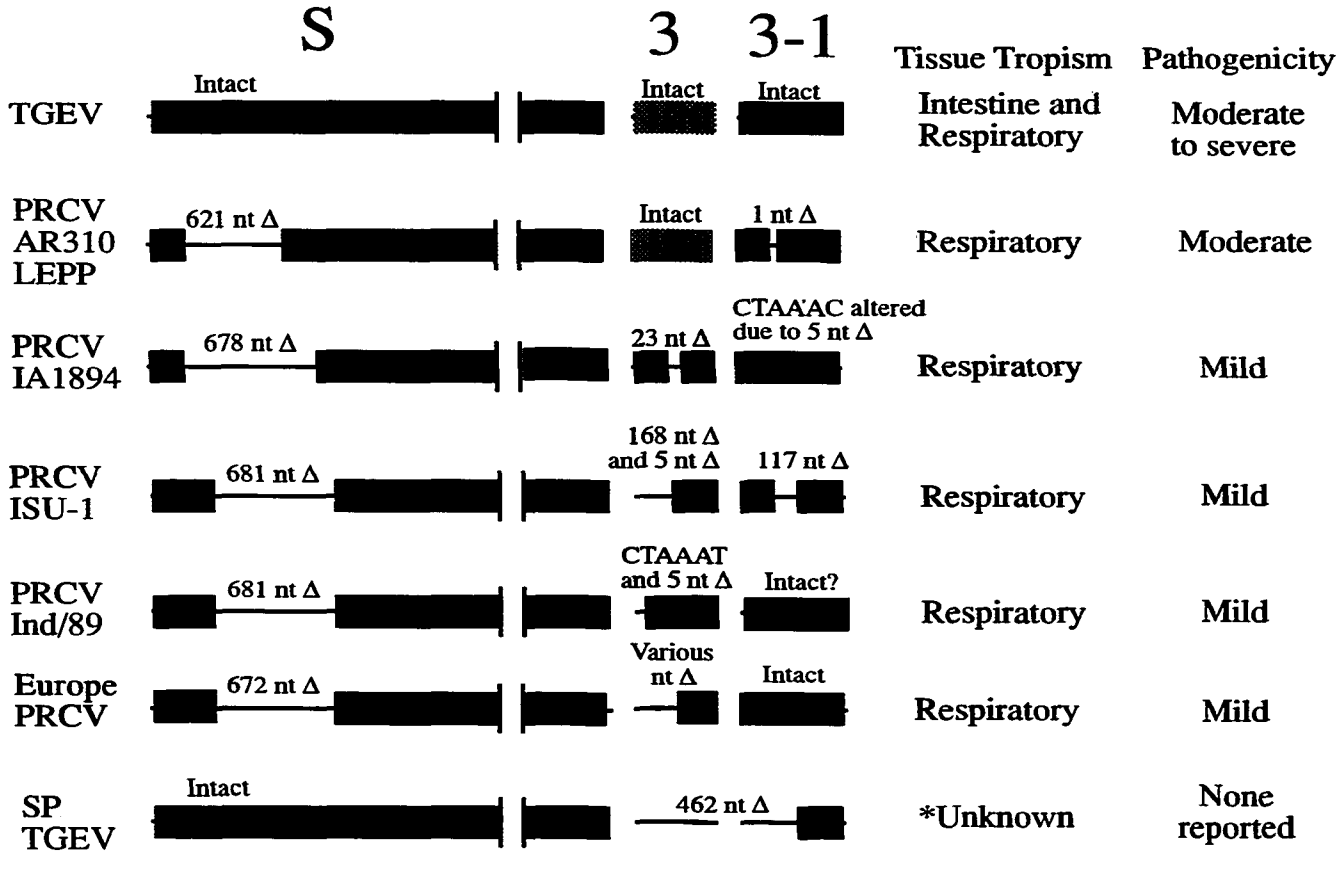


Figure 14. Schematic diagram showing the comparison of gene deletions, tissue tropism, and pathogenicity of TGEV and PRCV isolates. Heavy black lines indicate no deletions are present. Thin black lines indicate that deletions are present. Deletions are indicated as nucleotides (nt) deleted (Δ). The nucleotide sequences of the TGEV isolate CHV and of the PRCV isolates AR310, LEPP, IA1894, and ISU-1 were determined in this study. The nucleotide sequences of the other PRCV isolates were previously determined (Ind/89, Wesley et al., 1991; 86/137004, Britton et al., 1990; and RM4, Rasschaert et al., 1990). *The tissue tropism of the SP TGEV isolate is unknown as SP TGEV does not replicate in villus enterocytes but rather in cells found in the lamina propria, and that the extent of SP TGEV replication in respiratory tissue is not known.

THE USE OF NONRADIOACTIVE cDNA PROBES TO DIFFERENTIATE PORCINE RESPIRATORY CORONAVIRUS AND TRANSMISSIBLE GASTROENTERITIS VIRUS ISOLATES

A paper to be submitted to the *Journal of Veterinary Diagnostic Investigations*

Eric M. Vaughn, Patrick G. Halbur, and Prem S. Paul

Abstract

My colleagues and I have developed two cDNA probes from the S gene of TGEV that can be used to differentiate between PRCV and TGEV isolates. The probe FP2 hybridizes with RNA from both PRCV and TGEV, whereas the probe FP1 hybridizes only with RNA from TGEV. Both FP1 and FP2 were amplified by PCR and labeled with digoxigenin-labeled dUTP. The digoxigenin-labeled FP1 and FP2 probes were used in a dot blot hybridization assay to differentiate between PRCV and TGEV isolates grown in cell culture, and specific hybridization was detected using an immunochemiluminescent detection method. The probes FP 1 and FP 2 were found to be specific for TGEV and/or PRCV RNA as they did not react with nucleic acid from two group A rotaviruses (OSU and Gottfried), porcine parvovirus, and three enteroviruses (groups 1, 2, 8c). My colleagues and I found that TGEV or PRCV RNA can be obtained from virus-containing lysates from infected cell cultures, or total RNA could be obtained from infected ST cell cultures that are displaying TGEV or PRCV CPE. When using TGEV genomic RNA from virus-containing cell culture lysates, my colleagues and I found that at least 10^5 pfu of TGEV could be detected. The immunochemiluminescent dot blot hybridization assay described in this study should be useful in laboratories that

are attempting to isolate PRCV and need a rapid, specific, and nonradioactive detection system to differentiate PRCV from TGEV. Also, this procedure should be useful in field studies to determine the prevalence of PRCV in swine herds.

Introduction

Porcine respiratory coronavirus (PRCV), a member of the family *Coronaviridae*, is antigenically related to transmissible gastroenteritis virus (TGEV) of swine (3, 4, 6, 9, 12, 16, 18, 19, 23). Porcine respiratory coronavirus is now thought to be a mutant of TGEV (3, 9, 13, 22). Porcine respiratory coronavirus was first isolated in 1984 (12) from pigs in Belgium that were seropositive for TGEV but did not have a history of clinical transmissible gastroenteritis (TGE). Since the initial isolation of PRCV, infections of swine in Europe with PRCV are widespread (4, 9, 8). Porcine respiratory coronavirus has also been isolated from swine in the United States (7, 11, 19, 23). However, the prevalence of PRCV in swine herds within the United States is not known.

There are several similarities between PRCV and TGEV. Both viruses have three major structural proteins: the surface spike (S) glycoprotein, the integral membrane (M) glycoprotein, and an internal nucleoprotein (N) (3, 9, 13, 23). Nucleotide sequence of PRCV isolates thus far studied show that they are closely related to TGEV (3, 13, 22). However, there are some striking differences in that PRCV isolates have a characteristic deletion in the 5' end of the S gene when compared to TGEV (3, 8, 13, 19, 22). Porcine respiratory coronavirus has a different tissue tropism than TGEV. Transmissible gastroenteritis virus replicates in both the respiratory and intestinal tissues and causes gastroenteritis (14), whereas PRCV replicates to high titers in lung tissue of swine and with little or no replication in the intestinal tissues and no evidence of gastroenteritis and villus atrophy (5, 7, 23).

Porcine respiratory coronavirus is antigenically related to TGEV in that polyclonal sera which neutralize TGEV also neutralize PRCV (4, 6, 12, 16, 23). Thus, conventional serological methods are not useful in determining if a swine herd with anti-TGEV antibodies has been infected with PRCV or TGEV. Anti-TGEV neutralizing monoclonal antibodies (MAbs) directed against the S glycoprotein readily neutralize PRCV, however, there are some non-neutralizing anti-TGEV MAbs directed against the S glycoprotein that can be used to distinguish between PRCV and TGEV isolates in a competitive binding assay (4, 6, 16, 18).

Of the European PRCV isolates that have had their nucleotide sequences published all have a 672-nucleotide deletion in the 5' end of the S gene (3, 13). The U.S. PRCV isolates Ind/89 and ISU-1 had a 681-nucleotide deletion present in the 5' end of the S gene (8, 19, 22). Recently, the PRCV isolate AR310 was shown to have a smaller S gene deletion of 621-nucleotides present (19). Hence, a cDNA probe that encompasses the region of the TGEV S gene that is characteristically deleted from PRCV isolates can be used in a nucleic acid hybridization to differentiate between PRCV and TGEV isolates. My colleagues and I have developed two cDNA probes on the basis of the nucleotide sequence of the S gene of TGEV that can be used to differentiate between PRCV and TGEV isolates (19). Other researchers have reported on the use of TGEV cDNA probes in a nucleic acid hybridization to differentiate between TGEV and PRCV isolates, however, these previously reported hybridization methods relied on using cDNA probes labeled with the radionuclide ^{32}P (1, 8, 17, 21). My colleagues and I report here a nucleic acid hybridization assay that uses a immunochemiluminescent detection method in order to differentiate between TGEV and PRCV isolates, thus avoiding the use of radioactive materials.

Materials and Methods

Virus isolation

The isolation of the PRCV isolates AR310, LEPP, and IA1894 has been previously described (19). The PRCV isolates PON, IA725, and IA588 were isolated from nasal swabs from swine from herds that had antibodies to TGEV but had not presented evidence of diarrhea, and thus were suspected of having a PRCV infection. Nasal swabs were collected and placed into one ml of MEM with 2 percent FBS and antibiotics and mixed for 20 seconds. Two hundred µl of the nasal swab MEM was inoculated onto ST cells. CPE was present on the first passage in ST cells for the PRCV isolates PON, IA725, and IA588. The PRCV isolates AR310, LEPP, and IA1894 were plaque purified a total of three times and stock virus was stored at -70°C. The PRCV isolates PON, IA725, and IA588 were not plaque purified for this study.

The Miller strain of TGEV was used as a standard TGEV strain in this study. The PRCV isolate ISU-1 was received as a plaque-purified preparation and was kindly provided by Dr. Howard Hill (Iowa State University Veterinary Diagnostic Laboratory) (7).

Total RNA isolation

ST cells were infected at a multiplicity of infection (moi) of approximately 0.1 PFU/cell with CHV TGEV, and the PRCV isolates AR310, ISU-1, IA1894, or LEPP. At 19 hours post infection, the medium was removed and the total RNA was isolated from the infected ST cell monolayers by a rapid guanidinium thiocyanate method (Stratagene, La Jolla, CA). The moi used for the PRCV isolates PON, IA725, and IA588 was unknown as these isolates were not plaque purified and not titered. Total RNA from the ST cell monolayers infected with the PRCV isolates PON, IA725, and

IA588 was isolated when the infected ST cell monolayers started showing approximately 25% CPE. Total RNA from uninfected ST cell monolayers was also isolated and used as a negative control. The RNA was washed with 70% ethanol and dissolved in DEPC-treated distilled water and stored at -70°C.

Dot blot hybridization assay

Ten μg of total RNA from the PRCV infected and uninfected ST cell monolayers was denatured with formaldehyde and formamide (15) and blotted to nylon membranes (Magna NT, Micron Separations Inc., Westboro, MA) using a 96-well hybridot manifold apparatus (Schleicher and Schull, Keene, NH). The membranes were washed in 10x SSC (1x SSC is 0.15 *M* NaCl plus 15 m*M* sodium citrate), allowed to air dry, and then baked at 80°C for 2 hours to fix the RNA. The membranes were prehybridized for 2 hours in a solution containing 50% formamide, 5x SSPE (1x SSPE is 0.18 *M* NaCl, 10 m*M* sodium phosphate, and 1 m*M* EDTA), 4x Denhardt's solution, 1.0% sodium dodecyl sulfate (SDS), and sonicated salmon sperm DNA (30 μg/ml) at 42°C. The cDNA probes used in the hybridization procedure were designated FP2 and FP1 (Figure 1). FP2 is a 2.28 kb PCR product that was amplified by the primers 101004 and 060704 (Table 1) and cut with the *Bam*HI restriction enzyme and cloned in the phagemid vector pKS+ (Stratagene, La Jolla, CA). FP1 is a 0.58 kb PCR product that was amplified by the primers 101004 and 100907 (Table 1) and cut with *Bam*HI and *Eco*RI restriction enzymes and cloned in the phagemid vector pKS+. FP2 encompasses the 5'-half of the TGEV S gene and thus will hybridize to both TGEV and PRCV S genes. However, FP1 encompasses the region of the TGEV S gene that is deleted from all PRCV isolates known thus far and will only hybridize with the S gene of TGEV. The FP1 and FP2 PCR products were originally amplified from a plasmid containing the Miller TGEV S

gene using the appropriate primers under the following parameters: 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 5 minutes at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C; followed by 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 5 minutes at 72°C in a DNA thermal cycler (Coy Corporation, Grass Lake, MI). To make cDNA probes the FP1 and FP2 PCR products were reamplified from their respective plasmids using the conditions described above. The FP1 and FP2 PCR products were then separated on a 2% NuSieve GTG (FMC Bioproducts, Rockland, ME) agarose gel and then purified from the agarose gel by the Magic PCR prep method (Promega, Madison, WI). Twenty-five ng each of FP1 and FP2 PCR products were labeled with digoxigenin-labeled dUTP in the presence of random hexamer primers and Klenow fragment of DNA polymerase I (Genius 1 Kit, Boehringer Mannheim, Indianapolis, IN). Unincorporated digoxigenin-labeled dUTP was removed by ethanol precipitation. The digoxigenin-labeled FP1 and FP2 probes were then heated to 100°C, cooled on ice, and then added to the appropriate prehybridization reaction and allowed to hybridize overnight. The membranes were washed once in 2xSSC-0.3% SDS at room temperature, twice in 2xSSC-0.3% SDS at 65°C, and once with 0.2x SSC at 65°C. The membranes were washed in buffer 1 (buffer 1 is 100 m*M* maleic acid and 150 m*M* NaCl, pH 7.5) for 1 minute, followed by a 30 minute incubation in buffer 2 (buffer 2 is 1% blocking agent). The membranes were then incubated for 30 minutes in buffer 2 containing 150 mU/ml of alkaline phosphatase labeled anti-digoxigenin conjugate. Unbound antibody conjugate was removed by washing the membranes twice in buffer 1 for 15 minutes. The membranes were equilibrated in buffer 3 (buffer 3 is 100 m*M* Tris-HCl, 100 m*M* NaCl, and 50 m*M* $MgCl_2$, pH 9.5) for 2 minutes. For immunochemiluminescent detection, the membranes were repeatedly passed through Lumi-Phos 530 (Boehringer Mannheim, Indianapolis, IN) and enclosed in plastic wrap.

Chemiluminescent signals were detected by exposing X-ray film (RX film, Fuji Photo Film Co., Japan) to the membranes.

Sensitivity of the nonradioactive probes

The titers of PRCV AR310 and Miller TGEV were determined by plaque assay as previously described (20). The PRCV isolate AR310, and the Miller strain of TGEV, were diluted in MEM to adjust to a virus concentration of 10^6, 10^5, 10^4, 10^3, 10^2, or 10^1 pfu per 100 µl. SDS and proteinase K were added to each dilution of PRCV or TGEV to a final concentration of 0.1% and 500 µg respectively, mixed, and incubated at 37°C for 30 minutes. The extracted viral RNA was then heated to 100°C for five minutes and then cooled on ice and blotted to nylon membranes as previously described (10) and then used in a dot blot hybridization assay as described above.

Specificity of the nonradioactive probes

To determine the specificity of the nonradioactive probes, cell culture lysates from two group A rotaviruses (OSU and Gottfried), three enterovirus isolates (groups 1, 2, and 8c), porcine parvovirus (NADL-2) were treated with SDS and proteinase K, boiled, and blotted to nylon membranes as previously described (10). The membranes were then used in a dot blot hybridization assay as described above.

Results

As expected, the probe FP2 detected the TGEV and PRCV mRNAs present in the total RNA isolated from the PRCV- and TGEV-infected ST cells (Figure 2). The probe FP1 detected only TGEV mRNA present in the total RNA isolated from the TGEV-infected ST cells. The FP1 and FP2 probes were shown to be specific as the probes

showed no reactivity to the rotavirus, enterovirus, or parvovirus nucleic acids (data not shown). Using RNA from virus-containing cell culture lysates showed that at least 10^5 pfu could of TGEV be detected (data not shown). The FP1 and FP2 probes did not hybridize to the RNA isolated from the uninfected ST cell culture. All of the PRCV isolates replicated and produced CPE in the ST cell line on the first passage, and this appears to be a typical characteristic of PRCV isolates.

Discussion

The probe FP2 described in this study was specific for TGEV and PRCV RNA in a immunochemiluminescent dot blot hybridization assay. Additionally, the probe FP1 was able to differentiate between PRCV and TGEV isolates in a immunochemiluminescent dot blot hybridization assay. This nucleic acid hybridization procedure takes advantage of the characteristic deletion in the 5' end of the S gene found in PRCV isolates. All PRCV isolates studied thus far have had large deletions in the 5' end of the S gene ranging from 621- to 678-nucleotides in length (3, 8, 13, 19, 22). The probe FP1 is a 0.58 kb probe that is within the 621-nucleotide deletion site found in the PRCV isolate AR310 (19).

The source of TGEV or PRCV RNA to be used in the above described immunochemiluminescent dot blot hybridization assay can be isolated in one of two ways. First, TGEV or PRCV RNA can be obtained from virus-containing lysates from infected cell cultures, or total RNA can be obtained from infected ST cell cultures that are displaying TGEV or PRCV CPE. When using RNA from virus-containing cell culture lysates, my colleagues and I found that at least 10^5 pfu of TGEV could be detected. Shockley et al. (17) calculated that $2x10^6$ virions of TGEV is equivalent to 25 pg of TGEV genomic RNA. Thus, the 10^5 pfu detected in this study would indicate that the

immunochemiluminescent dot blot hybridization assay described here is able to detect the equivalent of 1.25 pg of TGEV genomic RNA. Other researchers have reported that TGEV specific cDNA probes labeled with ^{32}P can detect from 25 to 200 pg of genomic TGEV RNA. This indicates that the immunochemiluminescent dot blot hybridization assay described here using digoxigenin-labeled cDNA probes is 20 to 160 times more sensitive than previous reports utilizing ^{32}P-labeled cDNA probes.

The probes used in this study were found to be specific. Also, my colleagues and I have found that when using the immunochemiluminescent dot blot hybridization assay procedure to detect TGEV or PRCV RNA in virus-containing cell lysates, a rather high background level was present. The high background generally was evident on exposure of the X-ray film to the nylon membranes for periods longer than one hour. Thus, it appears that isolating total RNA from infected ST cells that are showing CPE of PRCV or TGEV will give the best results in this procedure.

My colleagues and I have found that enterovirus contamination is a common occurrence in ST cell cultures when using nasal swabs to isolate PRCV from swine. Even though enterovirus causes a different CPE in ST cells than that produced by PRCV, enterovirus contamination should not be a problem in this assay as the cDNA probes used in this study were shown not to react to three different groups of enteroviruses (group 1, 2, and 8c). As reported by other researchers, it would be expected that the probe FP2 would detect nucleic acid from antigenically related coronaviruses, such as FIPV and CCV (1, 2, 21).

Though PRCV continues to be isolated from swine herds in the United States, the prevalence of PRCV is not known. As more researchers attempt to obtain PRCV isolates for field studies on the prevalence of PRCV or to further study the molecular characteristics of new PRCV isolates, the immunochemiluminescent dot blot

hybridization assay should be beneficial. The immunochemiluminescent dot blot hybridization assay described in this study provides a rapid, specific, and nonradioactive detection system to differentiate PRCV from TGEV.

Acknowledgments

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TABLE 1. TGEV S gene specific oligonucleotide primers used in PCR to amplify regions of the S gene of TGEV

Primer		
Name	Sequence	Nucleotide localization
060704	5' ggggatccGCAGTGCCACGAGTCCTATCAT 3'	2462-2483 [a]
101004	5' gggggatccAGAACTATAGGTAACCATTGG 3'	1678-1698 [b]
100907	5' gggggaaTTCTAATGTAGTCGCACGCAT 3'	2230-2250 [b]

[a] From the sequence of D. Rasschaert and H. Laude. J. Gen. Virol. (1987) 68:1883-1890.

[b] From the sequence of P. Britton and K.W. Page. Virus Res. (1990) 18:71-80.

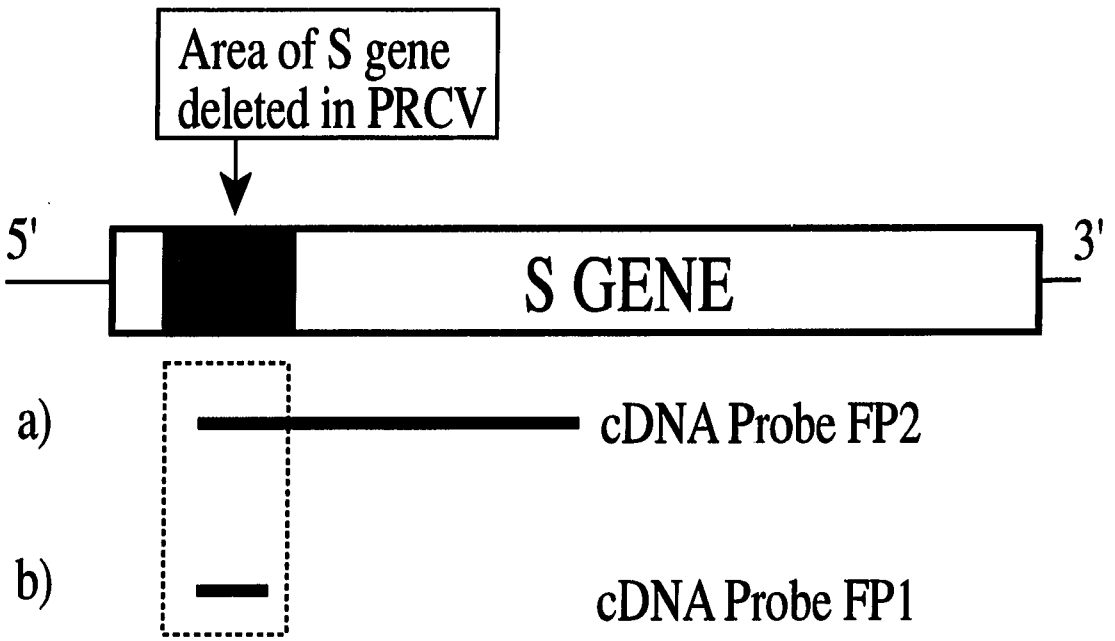
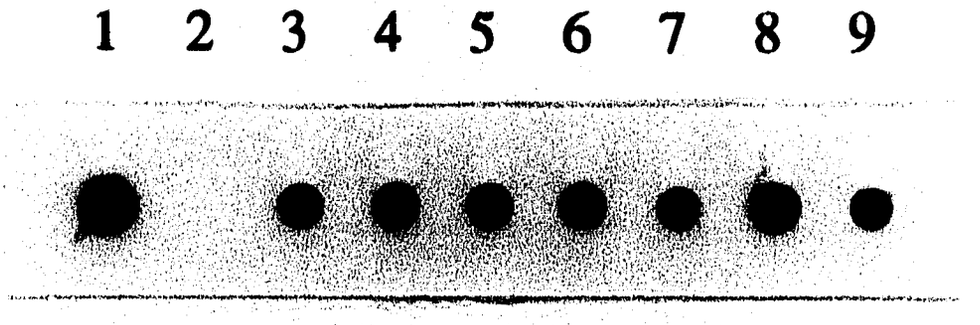


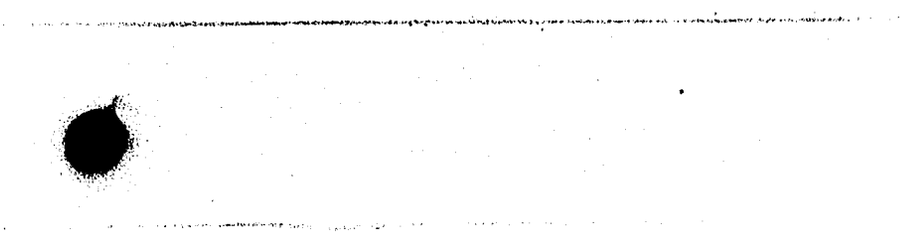
Figure 1. Schematic diagram of the S gene of TGEV with the characteristic deletion of PRCV shown. a) Probe FP2 used to detect the S gene of both TGEV and PRCV in a dot blot hybridization assay. FP2 was amplified by the primers 101004 and 060704 using a template of FP2 cloned in pKS+. b) Probe FP1 used to detect the S gene of TGEV in a dot blot hybridization assay. FP1 was amplified by the primers 101004 and 100907 using a template of FP1 cloned in pKS+. Dashed line represents the area that is deleted in PRCV.

Figure 2. Dot blot hybridization assay using the digoxigenin-labeled FP1 and FP2 cDNA probes and immunochemiluminescent detection of specific hybridization. A) The cDNA probe FP2 was labeled with digoxigenin-labeled dUTP and was hybridized with the total RNA from ST cell cultures that were infected with Miller TGEV (1), uninfected (2), or infected with ISU-1 (3), AR310 (4), IA1894 (5), LEPP (6), PON (7), IA725 (8), and IA588 (9). Note that the cDNA probe FP2 hybridized with total RNA from both TGEV- and PRCV-infected ST cell cultures. B) The cDNA probe FP1 was labeled with digoxigenin-labeled dUTP and was hybridized with the total RNA from ST cell cultures that were infected with Miller TGEV (1), uninfected (2), or infected with ISU-1 (3), AR310 (4), IA1894 (5), LEPP (6), PON (7), IA725 (8), and IA588 (9). Note that the cDNA probe FP1 hybridized only with total RNA from TGEV-infected ST cell cultures; the total RNA from PRCV-infected ST cell cultures was not detected with the cDNA probe FP1.

A



B



GENERAL CONCLUSIONS

In the first study, my colleagues and I isolated and partially characterized three new isolates of PRCV, designated AR310, LEPP, and IA1894. These PRCV isolates showed a selective tropism for respiratory tissue and were antigenically related to TGEV. Polymerase chain reaction (PCR) amplification of the 5'-half of the S genes of the three PRCV isolates indicated that a large deletion, characteristic of PRCV, was present. Using cDNA probes specific for the TGEV S gene, the PCR products were shown to be specific in a Southern blot. The three new PRCV isolates were shown to vary in S gene deletion size and pathogenicity. These new PRCV isolates should serve as important tools in gaining a better understanding of the pathogenesis of coronavirus infections.

The PRCV isolate AR310 has been shown to cause interstitial pneumonia in gnotobiotic pigs (Halbur et al., 1993). Using five-week-old SPF pigs, PRCV isolates AR310, LEPP, and IA1894 were shown to exhibit a tropism for respiratory tissue with no detectable replication in intestinal tissue (Halbur et al., 1994). Also, the pathogenicity of the PRCV isolates AR310, LEPP, and IA1894 was shown to vary. The PRCV isolates AR310 and LEPP induced moderate bronchointerstitial pneumonia in five-week-old SPF pigs, whereas the PRCV isolate IA1894 induced very mild bronchointerstitial pneumonia (Halbur et al., 1994).

Porcine respiratory coronavirus is believed to have originated from TGEV. Although the mechanism of the deletion occurring in the S gene of PRCV is not known, RNA recombination with polymerase jumping is a possible mechanism (Laude et al., 1993; Sanchez et al., 1992). Whether immune pressure plays a role in the deletion of the S gene in PRCV is not known. The PRCV isolate AR310 is the first PRCV strain to be isolated from intestinal tissue. The herd in which AR310 was isolated had been vaccinated with a commercial TGEV vaccine and immune pressure may have played a

role in the development of the S gene deletion of AR310. However, it should be noted that TGEV was also isolated from other pigs in the same herd from which AR310 was isolated. Whether the original AR310 intestinal homogenate contained a mixture of TGEV variants, or the S gene deletion of AR310 occurred during cell culture adaptation is under study at this time. Porcine respiratory coronavirus can cause a viremia (Cox et al., 1990a) and a possible explanation for the isolation of PRCV AR310 from intestinal tissue homogenates may have been due to a PRCV viremia at the time of tissue collection.

From information gained from the first study, my colleagues and I developed two cDNA probes from the S gene of TGEV that can be used to differentiate between PRCV and TGEV isolates. The probe FP2 hybridizes with RNA from both PRCV and TGEV, whereas the probe FP1 hybridizes only with RNA from TGEV. Both FP1 and FP2 were amplified by PCR and labeled with digoxigenin-labeled dUTP. The digoxigenin-labeled FP1 and FP2 probes were used in a dot blot hybridization assay to differentiate between PRCV and TGEV isolates grown in cell culture, and specific hybridization was detected using a immunochemiluminescent detection method. The probes FP1 and FP2 were found to be specific for TGEV and/or PRCV RNA as they did not react with nucleic acid from two group A rotaviruses (OSU and Gottfried), porcine parvovirus, or three enteroviruses (groups 1, 2, 8c). My colleagues and I found that TGEV or PRCV RNA can be obtained from virus-containing lysates from infected cell cultures, or total RNA could be obtained from infected ST cell cultures that are displaying TGEV or PRCV CPE. However, the best results were obtained by using total RNA from infected ST cells. When using TGEV genomic RNA from virus-containing cell culture lysates, my colleagues and I found that at least 10^5 pfu of TGEV could be detected. The immunochemiluminescent dot blot hybridization assay described here should be useful in

laboratories that are attempting to isolate PRCV and need a rapid, specific, and nonradioactive detection system to differentiate PRCV from TGEV.

In the second study, the nucleotide sequence from the S genes, the 3/3a genes, and the 3-1/3b genes of four PRCV isolates was determined and compared to other PRCV and TGEV isolates. Two of the PRCV isolates, AR310 and LEPP, were shown to be more pathogenic than another PRCV isolate IA1894 in a five-week-old specific pathogen free pig model (Halbur et al., 1994). All four of the PRCV isolates had a large inframe deletion in the 5' end of the S gene. The PRCV isolates AR310 and LEPP both had identical S gene deletions of 621-nucleotides starting 47 nucleotides after the S gene start site. The PRCV isolates, AR310 and LEPP, have the smallest deletions found thus far in the 5' end of the S gene (621-nucleotides). Until now, the size of the S gene deletions for other PRCV isolates from Europe and the United States has ranged from 672- to 681-nucleotides (Britton et al., 1990; Jackwood et al., 1992; Rasschaert et al., 1990; Wesley et al., 1991b). The PRCV isolate IA1894 had a 678-nucleotide deletion 44 nucleotides after the S gene start site, whereas the PRCV isolate ISU-1 had a 681-nucleotide deletion 62 nucleotides after the S gene start site. Even though the deletions in the 5' end of the S gene of PRCV AR310 and PRCV LEPP are smaller than that of other PRCV isolates, the area deleted encompasses the S protein amino acid residues of 92, 94, 218, and 219 that were predicted by Sanchez et al. (1992) to be important in predicting tissue tropism of PRCV and TGEV isolates.

Analysis of the 3/3a genes of the four PRCV nucleotides isolates showed a high degree of variability. The 3 gene of the PRCV isolates AR310 and LEPP was preceded by a CTAAAC leader RNA binding site and the 3 gene was predicted to yield a protein of 72 amino acids, the same size as that of the virulent Miller strain of TGEV. The 3a gene of the PRCV isolate IA1894 was preceded by a CTAAAC leader RNA binding site and

the 3a gene was predicted to yield a truncated protein of 53 amino acids due to a 23-nucleotide deletion in the 3a gene. The CTAAAC leader RNA binding site and ATG start codon of the 3 gene of the PRCV isolate ISU-1 was removed due to a 168-nucleotide deletion. Analysis of the 3-1/3b genes of the four PRCV nucleotides isolates also showed a rather high degree of variability. The 3-1 gene of the PRCV isolates AR310 and LEPP was preceded by a CTAAAC leader RNA binding site but was found to have an one-nucleotide deletion that was predicted yield a truncated (63 amino acids versus 244 amino acids) 3-1 protein. The last two nucleotides of the CTAAAC leader RNA binding site preceding 3-1 gene of the PRCV isolate IA1894 were removed by a five-nucleotide deletion but the 3-1 gene was predicted to encode for a protein of 244 amino acids. The 3-1 gene of the PRCV isolate ISU-1 was preceded by a CTAAAC leader RNA binding site but was found to have a 117-nucleotide inframe deletion that was predicted to yield a smaller (205 amino acids versus 244 amino acids) 3-1 protein. The fact that the PRCV isolates AR310 and LEPP were more pathogenic than the PRCV isolate IA1894 indicates that the presence of an intact 3 gene may be important in determining the pathogenicity of PRCV isolates.

Of significance, the PRCV isolates AR310 and LEPP are the first to have been found to have an intact and functional 3 gene. Surprisingly, the 3-1 gene coding region of the PRCV isolates AR310 and LEPP contained a one-nucleotide deletion is predicted to cause a frameshift in the ORF resulting in a truncated 3-1 protein of 64 amino acids. Whether this truncated 3-1 protein would still be functional is not known, but it may be possible that this truncated 3-1 protein may still have the necessary domains to be functional. The sequence information from all other PRCV isolates previously sequenced have shown that the 3 gene has deletions or alterations that render the 3 gene nonfunctional. However, the 3-1 gene of all other PRCV isolates shows that the 3-1

gene is preceded by a CTAAAC leader RNA-binding site and the following ORF was intact. Thus, the PRCV isolates previously reported on have displayed two certain characteristics typical of PRCV. First, previously described PRCV isolates displayed the characteristic of a large deletion in the 5' end of the S gene ranging from 672- to 681-nucleotides (Britton et al., 1990; Jackwood et al., 1992; Rasschaert et al., 1990; Wesley et al., 1991b). Second, these previously described PRCV isolates all have altered or partially deleted CTAAAC leader RNA-binding sites that result in the 3 gene being nonfunctional (Page et al., 1991; Rasschaert et al., 1990; Wesley et al., 1991b). Additionally, previously studied PRCV isolates, with the exception of Ind/89, all have deletions ranging from 22- to 36-nucleotides present in the putative 3 gene (Page et al., 1991; Rasschaert et al., 1990). The PRCV isolates AR310 and LEPP can thus be described as "uncharacteristic" PRCV isolates in that they have a smaller S gene deletion (621-nucleotides) and the 3 gene is predicted to be intact and functional and yet these isolates exhibit a tropism for respiratory tissue.

As the sequence information from the PRCV isolate IA1894 indicates, this particular isolate is more in keeping with the previously described PRCV isolates, in that PRCV IA1894 had a larger S gene deletion (678-nucleotides) and had a 23-nucleotide deletion in the 3a gene that would result in a truncated 3a protein. However, the 3b gene of the PRCV isolate IA1894 was preceded by an altered CTAAAC leader RNA binding site but the ORF was found to be intact and nearly identical to that of TGEV. This would mean, with the exception of the 23-nucleotide deletion present in 3a gene, that the PRCV IA1894 mRNA 3 is like that of Purdue and FS772/70 strains of TGEV in which the mRNA 3 is thought to code for both 3a and 3b proteins (Britton et al., 1990; Kapke et al., 1988).

The PRCV isolate ISU-1 also fits into the category of previously described PRCV isolates since PRCV ISU-1 had a larger S gene deletion (681-nucleotides) and had its 3 gene altered to a psuedogene due to a 168-nucleotide deletion in the 3 gene that removed both the CTAAAC leader RNA-binding site and the ATG start site of the 3 gene. Additionally, the 3-1 gene of PRCV ISU-1 was found to have a 117-nucleotide inframe deletion at amino acid residue 32, that would effectively remove 39 amino acid residues out of a possible 244 amino acid residues found in the intact 3-1 protein. Whether this 117-nucleotide inframe deletion in the 3-1 gene of PRCV ISU-1 would still yield a functional protein is not known. Since both the genes 3 and 3-1 of the PRCV isolate ISU-1 are altered, it exhibits the same genetic characteristics as the SP mutant of TGEV which is lacking the entire 3 gene and part of the 3-1 gene (previously described as regions A and B, respectively) due to a 462-nucleotide deletion (Wesley et al., 1990a). Using S1 nuclease analysis, Wesley et al. (1990a) demonstrated that the SP TGEV S gene was similar to that of virulent TGEV and contained no large deletions in the SP S gene characteristic of PRCV. The SP TGEV was not able to replicate in the villus epithelium of the gut of neonatal pigs and thus did not cause enteric disease. However, using IFA the SP TGEV was shown to replicate in the cells of the lamina propria of neonatal pigs infected with SP TGEV. It is not known if the SP TGEV was able to replicate in the lungs and other respiratory tissue of SP TGEV infected pigs. The SP TGEV was able to replicate in ST cells, but exhibited a SP morphology. The lack of intact 3 and 3-1 genes in PRCV ISU-1 does not affect its ability to replicate in ST cells or its plaque morphology, as PRCV ISU-1 can reach titers exceeding 10^6 pfu/ml and the plaque size is approximately 5 mm (data not shown). The lack of an intact 3a gene does not affect the ability of PRCV IA1894 to replicate in ST cells as it can reach titers exceeding 10^6 pfu/ml and the plaque size is approximately 5 mm (data not shown). In a

similar manner, the same can be said for the PRCV isolates AR310 and LEPP as these isolates readily grow to titers exceeding 10^6 pfu/ml and the plaque size is approximately 5 mm (data not shown). Wesley et al. (1990a) proposed that one of the functions of the 3 and 3-1 genes was determination of plaque size in cell culture. Since the PRCV isolate ISU-1 is missing the 3 gene just like all other PRCV isolates (except AR310 and LEPP), but has a smaller 3-1 protein and can still form large plaques in cell culture, the ability of the PRCV isolate ISU-1 to form large plaques may indicate that its smaller 3-1 protein is still functional. The 3-1 gene may be responsible for determining plaque size in cell culture as the SP TGEV mutant was lacking this region, and was not able to produce large plaques in cell culture.

Studies in our laboratory (Halbur et al., 1994), have shown that the PRCV isolates AR310 and LEPP are more virulent in five-week-old SPF pig model than the PRCV isolate IA1894. Wesley et al. (1990a) have hypothesized that the role of the 3 and 3-1 genes of TGEV are involved in pathogenesis and virulence. This hypothesis can be extended to PRCV. The fact that the PRCV isolates AR310 and LEPP both have an intact 3 gene, and the fact that these two isolates have been shown to cause more extensive lung lesion development and are more pathogenic than the typical PRCV isolate IA1894, supports the hypothesis that the 3 gene is important in the virulence of both TGEV and PRCV.

It is believed that PRCV evolved from TGEV. The PRCV isolates AR310 and LEPP will be useful in understanding the origins of PRCV as they may represent intermediate isolates in the evolution of PRCV from TGEV.

In this study, my colleagues and I describe unique PRCV isolates that have varying locations and sizes of S gene deletions. The 3 gene was shown to be intact for the PRCV isolates AR310 and LEPP, but the 3-1 gene was predicted to code for a

truncated 3-1 protein due to a frameshift caused by a one-nucleotide deletion. The PRCV isolates AR310 and LEPP were shown to be more pathogenic in five-week-old SPF pigs than the PRCV isolate IA1894.

The PRCV isolate IA1894, except for a 23-nucleotide deletion in the coding region of 3a gene, had a mRNA 3 that was revealed to be very similar to that to that of the virulent TGEV isolates FS772/70 and Purdue. These two TGEV isolates have the 3a gene and the 3b gene present on single mRNA species and the 3b protein is assumed to be expressed from the functionally bicistronic mRNA 3 (Britton et al., 1990; Kapke et al., 1988; Laude et al., 1993). Since the PRCV isolate IA1894 has been determined to be less pathogenic than the PRCV isolates AR310 and LEPP, and yet the mRNA 3 of PRCV IA1894 is very similar (with the exception of the 23-nucleotide deletion) to that of two virulent TGEV isolates, this suggests that the 3 gene is important in determining the virulence of PRCV and TGEV isolates. Specifically, the region of the 3/3a protein that is deleted from IA1894 and yet is still conserved in the PRCV isolates AR310 and LEPP and the virulent TGEV isolates CHV, PP3, FS772/70, and Purdue is a region of the four amino acid residues KLGL. These four amino acid residues may comprise an important motif or domain in the 3/3a protein that is essential for virulence in the animal host. The function of the 3/3a protein and its role in virulence needs to be clarified with further study.

Future studies to clarify the role of the 3/3a gene in virulence of PRCV and TGEV isolates would best be addressed by the correction of 3/3a gene defects by RNA recombination. Also, the use of RNA recombination to introduce a TGEV-like S gene to repair the deleted S gene regions of PRCV isolates would also be useful to further clarify the role of the S gene and its smaller S protein product in the change of tissue tropism of PRCV from the enteropathogenic TGEV. The PRCV isolates AR310, LEPP, and

IA1894 would be excellent candidates for future experiments to correct 3/3a, 3-1/3b, and S gene deletions. For example, if the S gene deletion of AR310 or LEPP was corrected to a TGEV-like S gene, would the S gene-corrected AR310 or LEPP become enteropathogenic? Additionally, if the PRCV isolate IA1894 had the 23-nucleotide deletion present in the 3a coding region corrected, would the 3a gene-corrected IA1894 cause just as severe lung lesions as AR310 or LEPP in the five-week-old pig model? Furthermore, if the S gene deletion of the 3a gene-corrected IA1894 was corrected by RNA recombination would the S gene-corrected-3a gene-corrected IA1894 become an enteropathogenic TGEV isolate?

From the present study, my colleagues and I can confirm, as have others, that the 3/3a gene of both TGEV and PRCV isolates is highly variable. In addition, the 3-1/3b genes of at least some PRCV isolates appears to be variable also. The extent of deletions present in the PRCV isolates AR310, LEPP, IA1894, ISU-1 allows for some intriguing speculation as to the relationship of the S, 3/3a, and 3-1/3b genes in PRCV isolates. All previously analyzed PRCV isolates have been found to have large deletion in the 5' end of the S gene accompanied by an altered or deleted 3/3a gene, while the 3-1/3b gene has remained intact. In this study, my colleagues and I have reported on additional PRCV isolates that do have large S gene deletions. However, the genes 3/3a and 3-1/3b were more variable in that two of the PRCV isolates (AR310 and LEPP) are predicted to have intact 3 proteins combined with truncated 3-1 proteins of 64 amino acid residues; another PRCV isolate (IA1894) is predicted to have a truncated 3a protein of 55 amino acid residues combined with an intact 3-1 protein; and the PRCV isolate (ISU-1) has the entire 3 gene missing combined with a smaller 3-1 protein of 205 amino acid residues. It has been suggested by Laude et al. (1993) that there is a possibility of a functional link between the 3/3a and S gene alterations of PRCV isolates that may be complementing

each other. With the information presented here, the complementation of an altered 3-1/3b gene with an altered S gene may also be necessary. This may mean that in order to be a PRCV isolate, there must be a deletion in the S gene (to alter tissue tropism), accompanied by an altered 3/3a gene, an altered 3-1/3b gene, or both the 3/3a and 3-1/3b genes altered. In order to exhibit the characteristic tropism of PRCV, it may be that a large S gene deletion has to be accompanied by alterations in the 3/3a gene, the 3-1/3b gene, or both. A large deletion in the S gene may not be compatible without these alterations. Until the polymerase region of the coronavirus genome from virulent and avirulent coronaviruses can be studied in depth, the area of the genome most likely to be involved in determining pathogenicity will be genes 3 and 3-1. No function has been assigned to these proteins as of yet.

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APPENDIX:

EXPRESSION OF THE S GLYCOPROTEIN OF TRANSMISSIBLE GASTROENTERITIS VIRUS IN INSECT CELLS

Eric M. Vaughn and Prem S. Paul

Introduction

Transmissible gastroenteritis (TGE) is a common and economically important disease of neonatal swine (Saif and Bohl, 1986). Transmissible gastroenteritis is characterized by severe diarrhea, dehydration, and high mortality in piglets under two weeks of age (Saif and Bohl, 1986). The transmissible gastroenteritis virus (TGEV), belongs to the *Coronaviridae* family of viruses. The transmissible gastroenteritis virus has three structural viral proteins, these being M, S, and N (Saif and Bohl, 1986; Garwes et al., 1976). The M protein is glycosylated with an apparent molecular mass (M_r) of 25 to 30 kDa and is associated with the viral envelope. The spike protein,S, has an apparent M_r of 200 kDa and is also glycosylated (Garwes et al., 1976). The N protein is not glycosylated, has an apparent M_r of 45 to 50 kDa , and is also associated with the binding of viral RNA and serves as a nucleocapsid (Britton et al., 1988a). The S protein is the viral protein that induces neutralizing antibodies (Garwes et al., 1978/79). In this paper, my colleagues and I report on the expression of the recombinant TGEV S protein for eventual functional and immunological studies.

The insect baculovirus *Autographa californica* (AcNPV) has been widely used as a helper-independent expression vector for high level foreign gene expression (Luckow and Summers, 1989; O'Reilly et al., 1992). Recombinant proteins expressed in the AcNPV system appear to undergo proper post-translational modification and transport in

insect cells (Luckow and Summers, 1989; O'Reilly et al., 1992). In this paper, my colleagues and I report on the construction of the S gene and expression of the entire S gene sequence in insect cells, and demonstration of that at least one of the major neutralizing epitopes is maintained.

Materials and Methods

Genomic RNA isolation

The gut-passaged virulent Miller strain of TGEV (National Veterinary Services Laboratory, Ames, IA) also referred to as CHV TGEV, was the TGEV strain used in this study. The swine testicular (ST) cell line was used to isolate and propagate the viruses in this study and was maintained as previously described (Zhu et al., 1990). Four-day-old ST cells in 75 cm^2 flasks (Costar, Cambridge, MA) were infected with a multiplicity of infection of approximately 0.1 pfu of CHV TGEV. The infected ST cell cultures were incubated at 37°C for 48 hours. After 48 hours, when extensive CPE had developed, the cultures were frozen and thawed three times and clarified by centrifugation at 1000 x g for 10 minutes. The virus containing supernatant was then mixed with NaCl and PEG-8000 to give a final concentration of 2.3% and 7% respectively and stirred overnight at 4°C. The virus was then pelleted by centrifugation at 100,000 x g for 30 minutes at 4°C. The virus pellet was resuspended in 10 ml of MEM and was then placed on top of a discontinuous sucrose gradient (10%-25%-40%) and centrifuged at 100,000x g for 90 minutes. The virus-containing band at the 25%-40% sucrose interface was collected. The virus was then pelleted at 100,000 x g for 30 minutes in MEM. The partially purified virus pellet was than resuspended in 500 µl of TNE (pH 9.0) with 1% SDS and 20 µg proteinase K, and incubated at 37°C for 30 minutes. The genomic RNA was then

extracted with phenol:chloroform and ethanol precipitated. The RNA was washed with 70% ethanol and stored in water at -70°C.

cDNA synthesis and PCR amplification

First strand cDNA was made from the genomic RNA from infected ST cells by avian myeloblastosis virus reverse transcriptase using random oligonucleotide primers (Invitrogen, San Diego, CA). The cDNA-RNA hybrids were amplified by PCR using Taq DNA polymerase (Boehringer Mannheim, Indianapolis, IN) using the following primers and cycles.

The 5'-half of the S gene (2.3 kb) of TGEV was amplified using the primers 010708 and 060704 under the following parameters: 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 5 minutes at 72°C; 30 cycles of 1 minute at 94°C, 1 minute at 48°C, and 3 minutes at 72°C; followed by 1 cycle of 1 minute at 94°C, 1 minute at 48°C, and 5 minutes at 72°C in a DNA thermal cycler (Coy Corporation, Grass Lake, MI). The 3'-half of the S gene (2.2 kb) of TGEV was amplified using the primers 060703 and 021208 under the conditions described above.

Construction the TGEV S gene

A schematic diagram of the construction of TGEV S gene is shown in Figure 1. After PCR amplification, the 5'-half of the S gene (2.3 kb) and the 3'-half of the S gene (2.2 kb) of TGEV were digested with *Eco*RI and *Bam*HI and were separated on a 2% NuSieve GTG (FMC Bioproducts, Rockland, ME) agarose gel and then purified from the agarose gel by the Magic PCR prep method (Promega, Madison, WI). The gel-purified PCR products were then cloned in the phagemid vector pKS+. The 5' and 3' termini of the two clones, designated CHV 1-2a (5' half of the S gene) and CHV 2-1 (3'

half of the S gene) were sequenced with universal and reverse primers for verification of the clones. The inserts from CHV 1-2a and CHV 2-1 were removed by digestion with *Eco*RI and *Bam*HI and were gel-purified as described above. Both inserts from CHV 1-2a and CHV 2-1 were digested with *Kpn*I and again gel-purified. The *Eco*RI/*Bam*HI/*Kpn*I digested inserts were then ligated into pKS+ that had been digested with *Eco*RI and *Bam*HI and dephosphorylated. One clone with a 4.3 kb insert was selected and the 5' and 3' termini of the clone, designated CHV S exp, were sequenced with universal and reverse primers for verification of the insert.

Generation of recombinant baculoviruses containing the S gene

The clone CHV S exp was digested with *Eco*RI and *Bam*HI and was gel-purified as described above and ligated into the *Eco*RI and *Bam*HI sites of the baculovirus expression vector pVL1392. A clone, designated pVL1392-S exp was selected and was purified by using the Qiagen column purification system. One hundred ng of the clone pVL1392-S exp was mixed with 50 ng of linearized and deleted AcNPV DNA in 25 µl of TE buffer and 25 µl of Lipofectin (Gibco BRL), mixed briefly, and incubated at room temperature for 30 minutes. The entire Lipofectin/DNA mixture was then added to Sf9 cells in a 6-well plate and incubated at room temperature for one hour. After one hour the Lipofectin/DNA mixture was removed and the Sf9 cells were then refed with Grace's insect medium with 10% FBS and antibiotics. The Sf9 cells were then incubated at 28°C for 5 days. After the 5 day incubation the cell supernatant containing recombinant S-baculoviruses was collected and stored at -70°C, and the Sf9 cells were fixed with methanol and screened by indirect immunofluorescence assay (IFA) to verify that recombination had occurred and that the S protein was being expressed. Once

recombination and S protein expression were confirmed, the recombinant S-baculoviruses were plaque purified three times.

Indirect immunofluorescence assay

Sf9 cells were grown in 6-well plates (Costar, Cambridge, MA) and infected with the recombinant S-baculovirus virus suspended in Grace's insect medium (Gibco BRL) with 10 % fetal bovine serum (FBS) and antibiotics, and incubated at 28°C. At 48 hours p.i., the medium was removed and the cells were fixed with methanol for 10 minutes. After removal of the methanol, the fixed cells were allowed to dry completely. The anti-TGEV S glycoprotein monoclonal antibody (MAb) 3H11 (Zhu et al., 1990) was incubated with the fixed cells for one hour at 37 C. The fixed cells were then washed and then stained with fluorescein-conjugated goat anti-mouse IgG (heavy and light chain) conjugate (Cappel Laboratories, Malvern, PA) for one hour at 37 C. The unbound conjugated antibodies were washed away with PBS, and glycerol:PBS (1:1) was added to each well. The plates were then examined for immunofluorescence. Uninfected Sf9 cells were processed in the same manner as above and served as negative controls.

Radioimmunoprecipitation assay

A RIP was utilized to determine the size and level of expression of the recombinant S glycoprotein in Sf9 cells. Sf9 cells in 6-well plates were infected at a m.o.i. of 0.1 pfu/cell with the recombinant S-baculovirus and incubated at 28°C. Uninfected Sf9 cells were used as a mock-infected control. At 24, 48, 72, and 96 hours p.i., the medium was replaced with methionine-free Grace's insect medium and the cells were incubated at 28°C for one hour. The methionine-free Grace's insect medium was replaced with fresh methionine-free MEM with 250 μCi/ml ^{35}S-methionine-cysteine

added. Two hours after the addition of the ^{35}S-methionine-cysteine, the cell monolayers scraped from the plastic flask and pelleted by centrifugation at 200 x g for 10 minutes. The cellular pellets containing labeled viral proteins and were then disrupted with 1 ml of lysis buffer (50 m*M* NaCl, 50 m*M* tris, 5m*M* EDTA, 1% Triton X-100, and 1m*M* phenylmethylsulfonyl fluoride). The lysis buffer and cellular pellet mixtures were mixed vigorously for one minute, and placed on ice for 3 minutes. The remaining cellular residues were removed by centrifugation. The lysates were stored at -20 C until needed. One hundred and fifty µl of the appropriate lysate was mixed with 4 µl of ascitic fluid containing the MAb 3H11 and incubated overnight at 4°C. Immune complexes were then collected by the addition of sepharose beads coated with protein A (Sigma, St, Louis, MO.), and incubated for one hour at room temperature. The antigen-antibody complexes/protein A sepharose beads mixtures were then washed three times with lysis buffer and then three times with deionized distilled water, resuspended in 50 µl sample buffer, and run on a SDS-polyacrylamide gel. Twenty-five µl of the appropriately labeled virus- or mock-infected interface were mixed with 25 µl of sample buffer (0.125M tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol) and placed in a boiling water bath for 5 minutes. The samples were electrophoresed at 15 mA constant current through a discontinuous 10% SDS-polyacrylamide gel. The ionic strength of the acrylamide gel and running buffer were 0.375M tris-HCl (pH 8.8) and 0.025M tris-HCl (pH 8.3), respectively. High and low molecular mass proteins were used as reference standards for determination of the molecular mass of viral proteins. The acrylamide gels were placed in fixation solution for one hour, then placed in autoradiography enhancer (NEN Research Products, Boston, MA) for 30 minutes, and then rehydrated in distilled water for 30 minutes. Then the acrylamide gels were dried and film (X-OMAT, Eastman Kodak Co., Rochester,

NY) exposed to the gels at -70 C for the production of autoradiographs. The molecular mass of the TGEV proteins were determined by running ^{14}C-labeled molecular mass markers on the same gel.

Results

An indirect immunofluorescence assay with the MAb 3H11 showed cytoplasmic fluorescence indicating that the recombinant S protein of TGEV was being expressed in the Sf9 cells (Figure 2). To verify that the recombinant S protein being expressed was of the correct molecular mass and to determine at which time maximum levels of the recombinant S protein were being expressed, a RIP was performed. The RIP showed that the MAb 3H11 had precipitated two protein bands (Figure 3). The S protein bands were determined to have a M_r of 180,000 and 160,000. The 180,000 protein is close to the expected size for the glycosylated S protein of 175,000 found intracellularly (Rasschaert and Laude, 1987). The 160,000 protein has the same M_r of the unglycosylated S protein (Rasschaert and Laude, 1987). The optimal level of S protein expression appeared to be at 48 hours p.i.

Discussion

Godet et al (1991) have previously expressed the S protein from the Purdue strain of TGEV insect cells and found that the predominate form of the recombinant S protein had a M_r of 175,000 which is similar in size to the 180,000 species my colleague and I identified. Godet et al. (1991) found a relatively low production (1% of the total protein content) of the TGEV S protein in insect cells and attributed this to the fact that insect cells are inefficient at expressing proteins of large size (200,000) and/or a high sugar content (28%). However, Godet et al. (1991) found that the recombinant S protein had

undergone trimerization like that found in the native S protein and many of the major neutralization epitopes were maintained. The discrepancy in size between the recombinant S protein and the native TGEV S protein (180,000 versus 200,000) could be attributed to the absence of terminal glycosylation in insect cells (Godet et al., 1991; Kuroda et al., 1990). Another possible reason for the size difference may be retarded processing due to the lack of efficient oligomerization in insect cells (Godet et al., 1991; Kuroda et al., 1991).

In conclusion, the ability of the MAb 3H11 to recognize the recombinant S glycoprotein in an IFA and a RIP indicates that at least one of the major neutralizing epitopes on the native TGEV S protein is present on the recombinant S protein. This indicates that if pigs were immunized with the recombinant S protein, some of the antibodies produced should be able to neutralize TGEV. Thus, the recombinant S protein expressed in insect cells should be useful in future studies of the functional and immunological role of the TGEV S protein.

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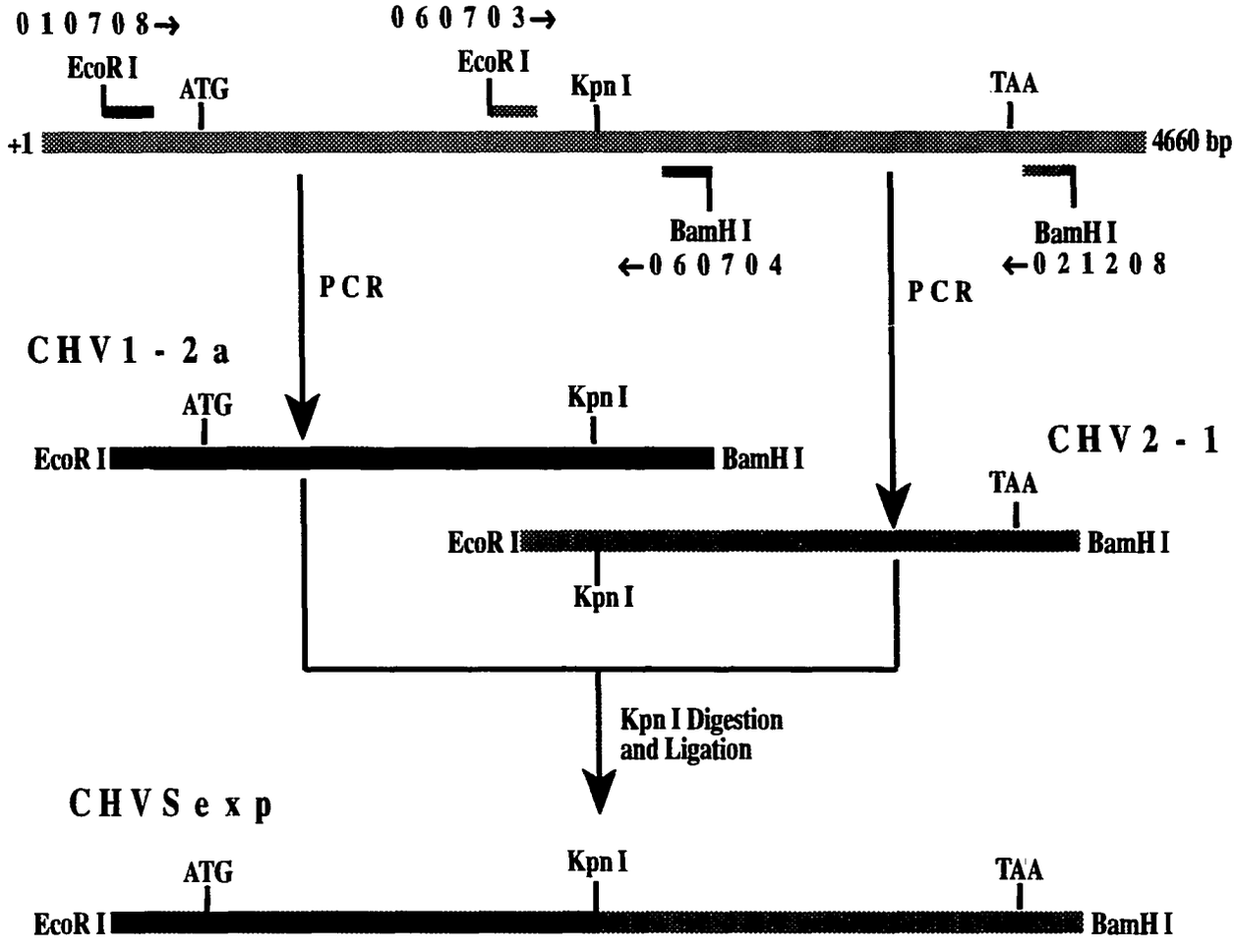
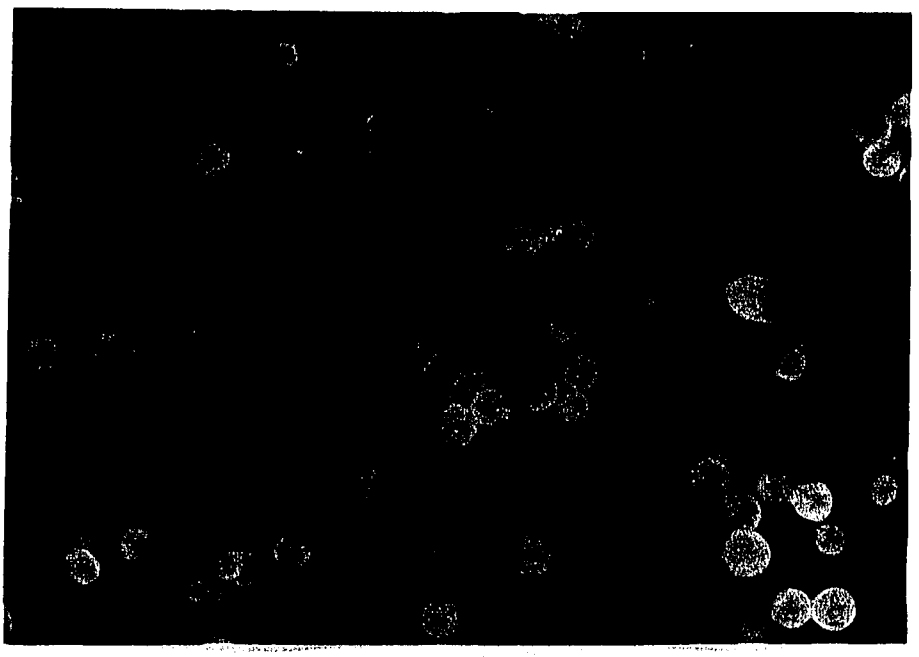


Figure 1. Construction of the S gene for expression in the baculovirus vector pVL1392. The 5'-half of the S gene was amplified by PCR with the primers 010708 and 060704, cloned, and designated CHV 1-2a. The 3'-half of the S gene was amplified by PCR with the primers 060703 and 021208, cloned, and designated CHV 2-1. The clones CHV 1-2a and CHV 2-1 were then digested with KpnI and ligated into pKS+ to form the clone CHV S exp.

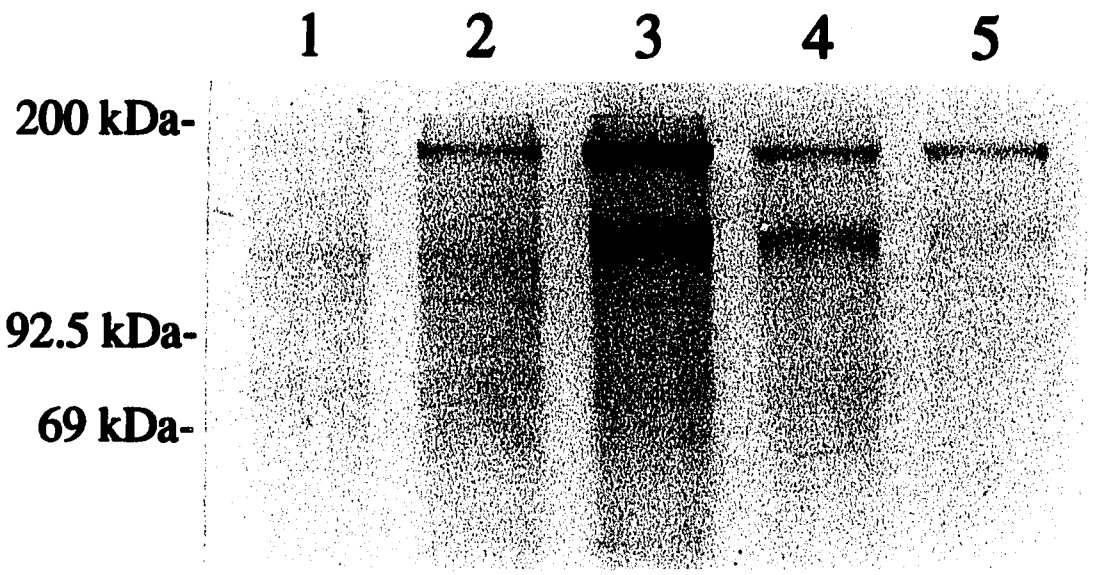
Figure 2. Immunofluorescent staining of recombinant S protein with the anti-TGEV S glycoprotein MAb MH11 in methanol fixed Sf9 cells infected with recombinant S-baculovirus (A), or uninfected (B).

A



B

Figure 3. Radioimmunoprecipitation of the recombinant S protein with the anti-TGEV S glycoprotein MAb MH11 from uninfected Sf9 cells (lane 1) and Sf9 cells infected with recombinant S-baculovirus for 24 (lane 2), 48 (lane 3), 72 (lane 4), 96 (lane 5) hours post infection.


1
2
3
4
5
200 kDa-
92.5 kDa-
69 kDa-